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Genetics in Aquaculture

Proceedings of the Sixteenth
U.S.-Japan Meeting on Aquaculture
Charleston, South Carolina
October 20 and 21, 1987

Ralph S. Svrjcek (editor)

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Ralph S. Svrjcek (editor)
Publications Unit
Northwest and Alaska Fisheries Science Centers

Panel Chairmen:
Conrad Mahnken, United States
Takeshi Nose, Japan

Under the U.S.-Japan Cooperative Program
in Natural Resources (UJNR)

November 1990

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PREFACE

The United States and Japanese counterpart panels on aquaculture were formed in 1969 under the United States–Japan Cooperative Program in Natural Resources (UJNR). The panels currently include specialists drawn from the federal departments most concerned with aquaculture. Charged with exploring and developing bilateral cooperation, the panels have focused their efforts on exchanging information related to aquaculture which could be of benefit to both countries.

The UJNR was begun during the Third Cabinet-Level Meeting of the Joint United States–Japan Committee on Trade and Economic Affairs in January 1964. In addition to aquaculture, current subjects in the program include desalination of seawater, toxic microorganisms, air pollution, energy, forage crops, national park management, mycoplasmosis, wind and seismic effects, protein resources, forestry, and several joint panels and committees in marine resources research, development, and utilization.

Accomplishments include: Increased communication and cooperation among technical specialists; exchanges of information, data, and research findings; annual meetings of the panels, a policy-coordinative body; administrative staff meetings; exchanges of equipment, materials, and samples; several major technical conferences; and beneficial effects on international relations.

Conrad Mahnken - United States
Takeshi Nose - Japan

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Assessment of Inbreeding and Its Implications for Salmon Broodstock Development*

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ABSTRACT

Inbreeding is an important part of any selection and breeding program designed to improve aquacultural broodstock. A decrease in freshwater and saltwater growth rate was noted in a strain of coho salmon, *Oncorhynchus kisutch*, undergoing selection to improve these traits for commercial production. Thus, an investigation was undertaken to estimate the level of inbreeding in this strain and to assess different approaches to alleviate problematic levels of inbreeding. Estimation of inbreeding level was conducted via pedigree analysis and change in heterozygosity of elctrophoretically detected serum proteins variants of odd- and even-year lines of coho salmon. The two methods of analysis indicated vastly different inbreeding levels. However, pedigree analysis, the more accurate of the two methods, estimated inbreeding levels not anticipated to cause the observed depression in growth traits. Two approaches, interstock crosses and crosses between parallel-selected lines, were assessed for alleviation of inbreeding problems. Both types of crosses decrease the level of inbreeding, but the performance of the two types of crosses differed greatly. Crosses between unrelated year classes of the selected stock showed positive heterotic effects, while the outcrosses with unrelated lines yielded negative heterotic effects. These results indicate that careful attention should be given to the selection of the founding populations from which broodstocks are developed and that subsequent breeding information be collected to produce pedigrees for population maintenance. Furthermore, the production of parallel "in-house" lines, may provide the best method of minimizing inbreeding without diluting selection gains.

Introduction

Inbreeding is integral to any selection and breeding program designed for the development of broodstock. Such programs generally deal with a "closed" population (i.e., migration into the population is eliminated) having a restricted breeding population size. Both of these factors result in increased inbreeding levels (Falconer 1981), where the magnitude will depend on the genetic characteristics of the population and the severity of the constraints imposed. Consequently, the factors that influence inbreeding must be integrated into the design of any program to develop genetically improved aquacultural stocks.

There has been a large amount of research concerning inbreeding and its effects on various traits in fish. For example, work with rainbow trout, *Oncorhynchus mykiss* (formerly *Salmo gairdneri*), has revealed that increased levels of inbreeding result in increased egg and fry mortality, increased numbers of abnormal fry, decreased early growth, and decreased fishery recovery (Kincaid 1976, 1983; Aulstad and Kittlesen 1971). Research with brook

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trout, *Salvelinus fontinalis*, has demonstrated a negative impact on weight owing to inbreeding (Cooper 1961). Ryman (1970) reported a decrease in recapture frequency in Atlantic salmon, *Salmo salar*, with increased levels of inbreeding. In general, the results of these studies suggest a negative impact on a variety of biological traits in the populations studied and, consequently, on production.

No studies have been published on the effects of inbreeding on Pacific salmon, *Oncorhynchus* spp., nor have any published reports dealt with the effects of inbreeding in conjunction with a selection and breeding program designed to develop a genetically improved stock for aquacultural purposes. To some degree, both of these deficiencies in information are being eliminated as Pacific salmon are used for captive culture. It is imperative that data be obtained on inbreeding in these species under defined programs to determine their response to selection.

Research Rationale

The University of Washington, Domsea Farms, Inc., and the Washington Sea Grant Program have been conducting a selection and breeding program with coho salmon, *O. kisutch*, to develop a broodstock for the marine net-pen industry in the State of Washington. The major objective of this nine year cooperative program has been to develop a broodstock with traits that are beneficial to the production of 300–350 g coho salmon for the "plate-size" salmon market.

The traits that have been emphasized for selective improvement are 1) freshwater growth, 2) smoltification, and 3) saltwater growth to harvest size. Genetic analyses of these traits in the stock employed by Domsea Farms revealed adequate variability to expect progress from selection (Iwamoto et al. 1982; Hershberger and Iwamoto 1984; Saxton et al. 1984).

Using estimated genetic values and considering that the facilities available to the program would only allow raising 40 families of 600 individuals or less, a selection scheme was designed to yield maximum response and to be useful in a commercial operation (Fig. 1). This scheme involved several different types of concurrent selection (e.g., family and individual) and used a selection index that incorporated heritability estimates, relative economic values, genetic correlations, and mean values on all the traits of interest. It was recognized early in the development of this scheme that potential inbreeding problems could arise from the rather severe limitation in breeding population size (only twenty individuals contribute to each generation). Consequently, breeding was conducted by a rotational line-crossing procedure (Fig. 2) to minimize the possibility of crossing within lines. On a theoretical basis, these steps should limit the change in inbreeding

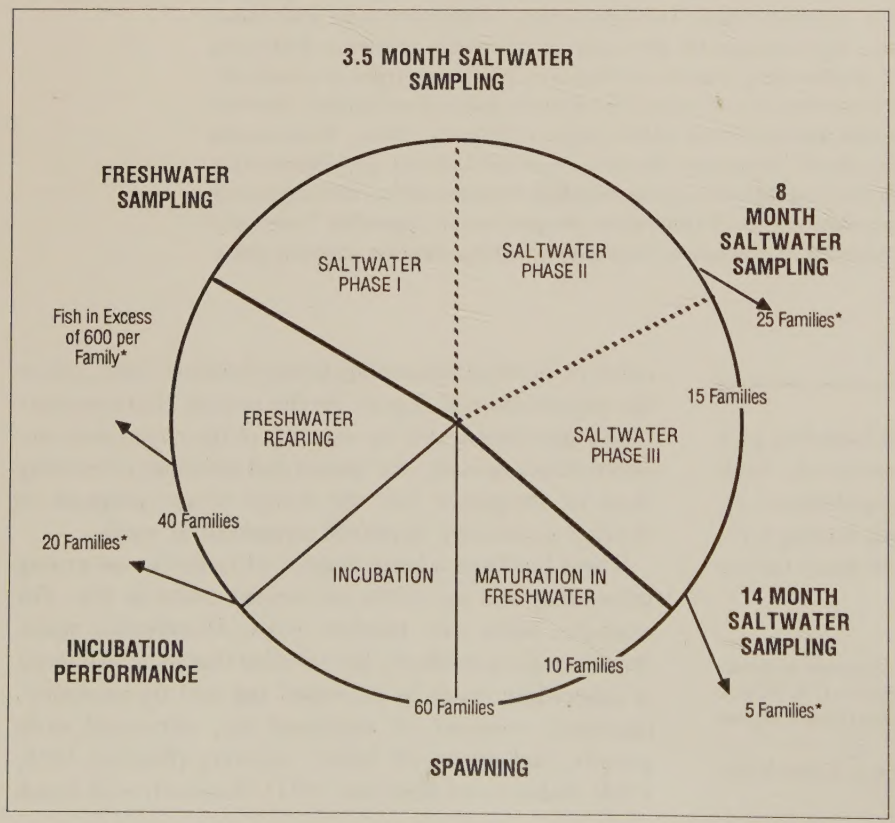

Figure 1
Diagram of the selection scheme used to develop coho salmon stocks for marine pen-culture. The entire cycle represents a two-year generation interval.

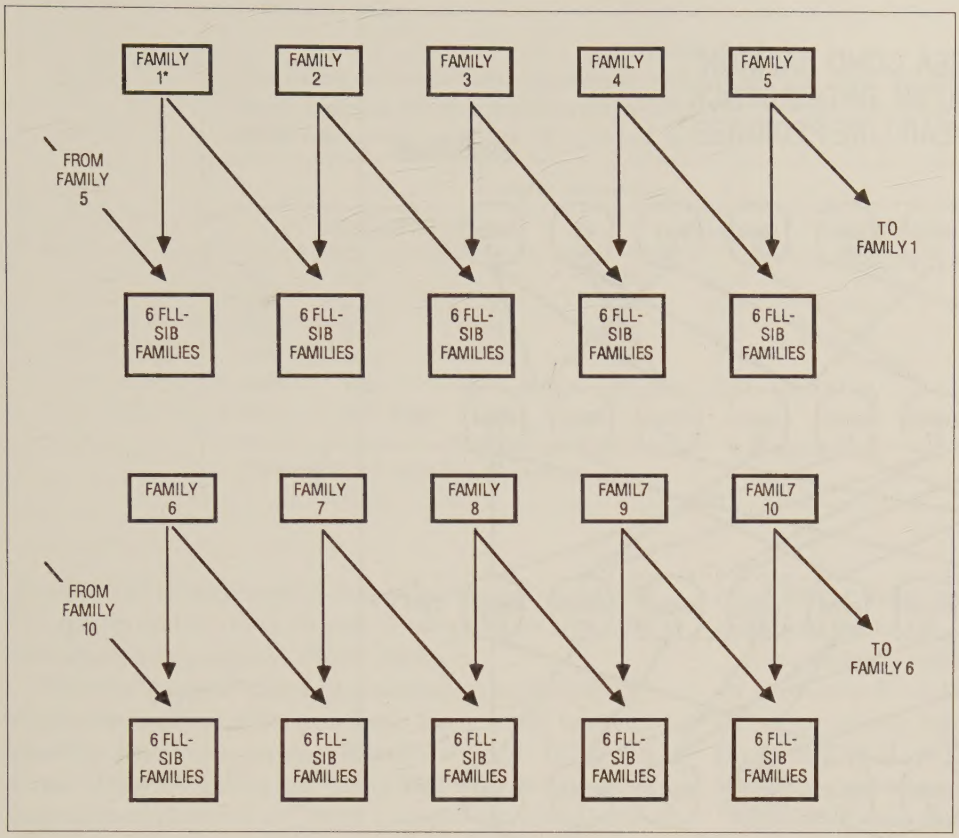

Figure 2
Diagram of the rotational line mating system used in crossing selected individuals. The asterisk indicates that each family cross is composed of six single-pair matings to form six double first-cousin families.

to about 1% per generation (Hershberger and Iwamoto 1984).

In 1983 (for the odd-year line) and 1984 (for the even-year line) a decrease in the growth of selected fish in saltwater was observed (Fig. 3). One possible explanation for this growth depression would be the accumulation of deleterious alleles through inbreeding. Even with the precautions taken in the design of the selection and breeding program, there were two potential sources of inbreeding that could not be quantitated. First, an unknown amount of inbreeding may have been introduced by selection and breeding that had occurred prior to use of this designed program. Second, because of some unexpected husbandry problems with raising fish to maturity there was a strong probability that a few families contributed disporportionately to the subsequent generations. Prior to the definition of pedigrees for the two lines, the importance of these factors was undeterminable.

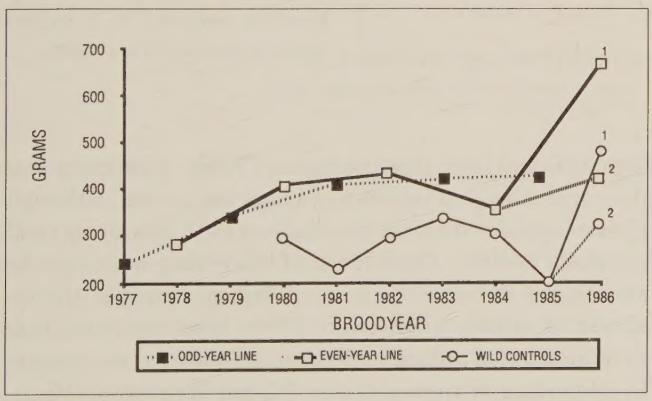

Figure 3
Average weight (grams) of selected broodstock and wild controls after 8 months rearing in marine net-pens. Weights for 1986 are given as unadjusted (1) and adjusted (2) for density differences that year. N = 1200–2200 for selected broodstock and N = 15–35 for wild controls.

As a result of these indications, studies were initiated to 1) determine the actual levels of inbreeding in the two lines and 2) define the best approach to eliminate inbreeding in the selected stocks.

Determination of Inbreeding Level

The level of inbreeding in each of the two selected lines (i.e., odd- and even-year) was determined by two different methods. First, pedigree analyses were employed to determine the coefficient of inbreeding (F) (Falconer 1981). Computation of this value is accomplished by tracing the

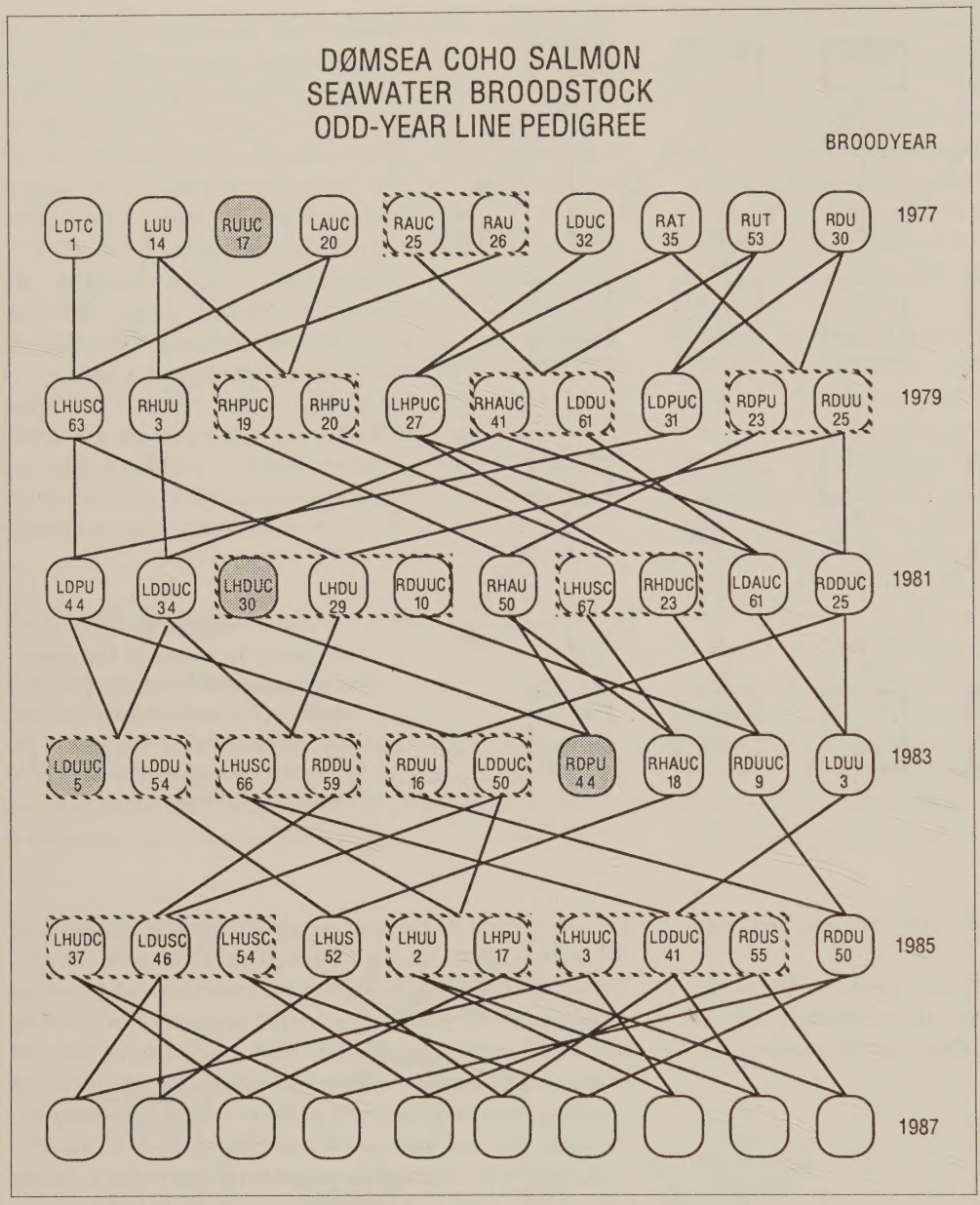

Figure 4
Pedigree of matings between selected families for the odd-year broodstock line (1978–1986). Families enclosed by a striped box are double first cousins.

pedigree back to common ancestors and determining the probability that a pair of alleles are identical by descent. Second, the change in genotype frequencies of electrophoretically analyzed protein differences were determined and the difference in heterozygote frequencies equated to an apparent inbreeding coefficient (Hartl 1980). Electrophoretic analyses were conducted on serum samples from 100–120 adult fish in each of four years (1977, 1978, 1985, and 1986). The electrophoretic procedures employed were those reported in Utter et al. (1970) for analysis of serum transferrins in coho salmon.

Construction of the pedigrees for the two lines of coho salmon revealed more closely related families than was originally anticipated (Fig. 4). Calculation of an inbreeding coefficient from these pedigrees (Table 1) indicates that the current level of inbreeding is not too severe, although the estimate for the next generation (1987 broodyear) will approach 8–10%. These levels of inbreeding would not be anticipated to cause the level of change found in the response of growth to selection. It has been estimated that in domesticated animals selection can balance an increase in inbreeding of approximately 2% per generation (Pirchner 1969). The estimated levels of inbreeding in coho salmon lines, to the point where apparent inbreeding depression was noted (1983 and 1984), are below this value. However, the coefficients reflect only the inbreeding since the program was initiated and do not provide a measure of prior inbreeding. Further, it is difficult to determine what

Table 1

Inbreeding estimates based on pedigree analysis for both odd- and even-year lines, and based solely on effective population size (*Ne*). The estimates are calculated assuming the initial inbreeding coefficient (*F*) is equal to 0.

Pedigree estimates						$\Delta F = (1/2N + 4)^a$	
	Odd-year	Control		Even-year	Control	Odd	Even
1977	0.00	—	1978	0.00	—	0.00	0.00
1979	0.00	2.50	1980	0.00	2.50	2.27	2.27
1981	0.32	4.71	1982	0.63	5.75	4.49	4.41
1983	2.34	8.68	1984	4.22	9.11	8.78	6.58
1985	4.86	11.00	1986	5.90	12.20	10.86	8.52
1987	8.34	13.79				12.88	

[a]Theoretical ΔF excluding sib-matings.

the effects of an incremental change in inbreeding may be in a species that has been recently developed from naturally reproducing populations (Soule 1980).

The second type of inbreeding assessment employed electrophoretic analysis of the transferrin locus, which has been shown to have three variant alleles (Utter et al. 1970) and is one of the few genetically variable protein loci found in coho salmon (Utter et al. 1980). Comparison of the genotype and gene frequency values in the original adult population with those from the fourth generation of selected stock (Table 2) revealed changes that would be anticipated in an inbred population (Falconer 1981); that is, there was a decrease in the frequency of heterozygotes and, with one exception, there was little change in the gene frequencies. Calculation of apparent inbreeding coefficients based on the frequency change in heterozygotes (Fig. 5) yields a much larger value than was obtained from the pedigree analyses (Table 1).

It is possible to rationalize the discrepancy in these values on two bases. First, there is evidence suggesting selective differences among the various alleles of the transferrin locus (Suzumoto et al. 1977; Pratschner 1977). The results of Pratschner's research indicated that fish with the "A" and "C" alleles were more resistant to challenges by *Vibrio* bacteria than those with the "B" allele, and Suzumoto et al (1977) found that the "A" allele imparted higher survival to BKD (bacterial kidney disease) challenge. If such selective pressures were applied to the selected coho salmon lines, analyses based on the genotype frequencies would tend to overestimate the inbreeding coefficient. The data from the current study support the hypothesis that fish with the "A" and "C" alleles have a selective advantage, and

Table 2

Observed transferrin gene and genotype frequencies in the odd- and even-year lines of coho salmon and their changes over four generations of selection (N = 100–120).

	Odd-year broodstock line								
	Genotype						Gene frequency		
Year	AA	AB	AC	BB	BC	CC	*f*A	*f*B	*f*C
1977	0.00	0.08	0.33	0.00	0.13	0.48	0.20	0.10	0.70
1985	0.05	0.03	0.08	0.00	0.18	0.68	0.10	0.10	0.80
Change	+0.05	−0.05	−0.25	+0.00	+0.05	+0.20	−0.10	0.00	+0.10
	Even-year broodstock line								
	Genotype						Gene frequency		
Year	AA	AB	AC	BB	BC	CC	*f*A	*f*B	*f*C
1978	0.10	0.05	0.45	0.05	0.25	0.10	0.35	0.20	0.45
1986	0.12	0.00	0.42	0.00	0.04	0.42	0.33	0.02	0.65
Change	+0.01	−0.05	−0.02	−0.05	−0.21	+0.32	−0.02	+0.18	+0.20

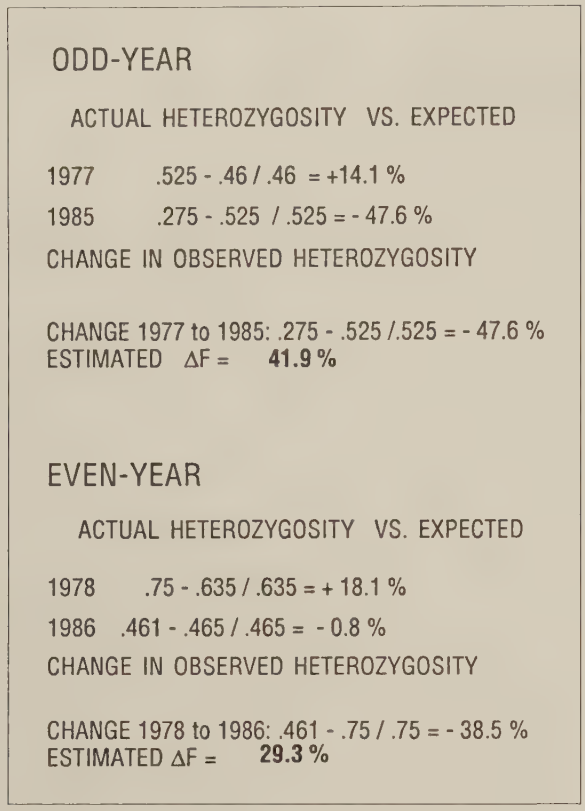
ODD-YEAR
ACTUAL HETEROZYGOSITY VS. EXPECTED
1977 .525 - .46 / .46 = +14.1 %
1985 .275 - .525 / .525 = - 47.6 %
CHANGE IN OBSERVED HETEROZYGOSITY
CHANGE 1977 to 1985: .275 - .525 /.525 = - 47.6 %
ESTIMATED ΔF = 41.9 %
EVEN-YEAR
ACTUAL HETEROZYGOSITY VS. EXPECTED
1978 .75 - .635 / .635 = + 18.1 %
1986 .461 - .465 / .465 = - 0.8 %
CHANGE IN OBSERVED HETEROZYGOSITY
CHANGE 1978 to 1986: .461 - .75 / .75 = - 38.5 %
ESTIMATED ΔF = 29.3 %

Figure 5
Estimates of the apprent inbreeding coefficients for the odd- and even-year lines based on the changes in observed and expected genotype frequencies of the coho transferrin alleles.

vibriosis is a common problem in the marine net-pen culture of salmon. The directed selection practiced on the stock may also have an epistatic effect on the transferrin locus. A tacit assumption made in the use of the genotype frequency relationship used to calculate an inbreeding coefficient is the absence of selection. Such an assumption is clearly not valid in this situation and may result in the inflation of the calculated value.

To summarize, it appears that pedigree analysis is the best approach to determine inbreeding levels in coho salmon. Thus, it would seem wise to assure that a selection and breeding program incorporates the mechanisms that define accurate pedigrees of the breeding population. Further, caution should be exercised in the use of genotype frequency changes to determine absolute values for inbreeding coefficients. The potential effects of direct and indirect selection must be determined for these values to be considered as valid measurements of inbreeding.

Elimination of Inbreeding

Although the apparent levels of inbreeding in the selected stocks of coho salmon were not large, two approaches to elimination of accumulated inbreeding were investigated: outcrossing between stocks and outcrossing between lines within stocks. Test crosses were made between the Domsea coho salmon stock and the hatchery stock of the University of Washington, and between the Domsea odd- and even-year parallel-selected lines. Progeny from these crosses were reared in conjunction with the broodstock line.

Table 3
The relative growth and survival of interstrain (Domsea × Univ. of WA) and intrastrain (Domsea odd- × even-year) crosses after 8 months rearing in marine net-pens. The weights and survivals have been standardized against the Domsea × Domsea (2 × 2) cross = 100. The index value is the cross-product of weight and survival/100. *N* = 8–45 for each cross.

Outcrossing schemes	Relative weight	Relative survival	Index
DOMSEA (D)	100	100	100
D × UW (♀ × ♂)	147	25	36.8
UW × D (♀ × ♂)	141	25	35.3
University of Wash. (UW)	55.1	21.4	11.7
DOMSEA line crosses	**Relative weight**	**Relative survival**	**Index**
DOMSEA (2 × 2)	100	100	100
DOMSEA (2 × 3)	116.1	150	174.5
DOMSEA (3 × 2)	101.4	225	174.1
DOMSEA (3 × 3)	128.7	100	128.7

It is apparent from the data (Table 3) that the progeny from the crosses derived from the Domsea intrastock crosses were superior to the interstock cross at the time of harvest. Although both of the University of Washington × Domsea hybrids were larger after eight months of saltwater rearing, relative to the Domsea controls, the overall survival of both the hybrids and the University of Washington fish was extremely poor under net-pen conditions. The high values reported reflect the survival of a few large hybrids which biased the weight measurements. The University of Washington × Domsea hybrids may not necessarily be indicative of all interstock crosses, but the results suggest that extensive hybrid testing may be necessary to identify a complementary stock. The Domsea intrastock hybrids, however, showed both good growth and greatly improved survival relative to controls. Maintaining "in-house" parallel selection lines may be a more efficient expenditure of effort relative to testing outcrosses. The "odd × even"

crosses would appear to be the method-of-choice for alleviating the inbreeding "load" while preserving selection gains.

Implications for Broodstock Development

The coho salmon stocks that have been developed as a result of this research program have, apparently, not yet reached a level of inbreeding which would result in a strong negative impact on their performance. The depression in growth observed in both lines appears to have been environmentally generated and subsequent generations have performed well (Fig. 3). However, analyses of inbreeding in these lines have demonstrated several areas requiring special consideration in the development of aquaculture broodstocks. Where possible, a selection and breeding program should be initiated with a large enough population size to completely address the combined needs of a reasonable selection differential and elimination of close familial relationships. Otherwise, definitive steps must be taken in the formulation of the selection and breeding program to minimize the accumulation of inbreeding from these factors.

Further, a broodstock program should be initiated from either an outbred population with an inbreeding coefficient (F) equal, or close to 0, or from a stock with a defined and well maintained pedigree. This would insure that the inbreeding level could be unquestionably determined and the effects of any increases could be well defined. In addition, research is needed to determine the response of aquacultural species recently derived from wild populations to an increase in inbreeding level. While the response of domesticated animals to increases in inbreeding has been quantitated to some degree (Pirchner 1969), there is no *a priori* method by which to predict the magnitude of responses in natural populations. As indicated by Gall (1987), the best information will be obtained from inducing high levels of inbreeding in such stocks and quantifying the effects. However, inbreeding effects observed in the progeny of sib-matings are indicative of, but not highly correlated with the performance of individuals with equal inbreeding levels produced through generations of matings.

Finally, it appears that using parallel selection in at least two separate lines of broodstock would be a valuable approach to incorporate into a selection and breeding program. This provides an additional data set with which to evaluate a selection program and also incorporates a mechanism that has the potential to eliminate inbreeding effects without the loss of advances made in the traits that are beneficial to aquaculture production.

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Chromosome Set Manipulation in Salmonid Fishes

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ABSTRACT

Techniques to manipulate chromosome sets and produce polyploid fishes or fishes with all the inheritance from the female or male parent have been exploited in aquaculture in recent years. Some of the principal applications of this work have been to produce sterile fish or to produce monosex populations. Three additional applications of chromosome set manipulation that we have explored in salmonids in our laboratory and in collaboration with other laboratories have been 1) increased survival in triploid hybrids; 2) the potential for gene transfer by "incomplete gynogenesis"; and 3) the generation of homozygous diploids and ultimately homozygous clones through androgenesis (all-paternal inheritance).

A number of researchers have demonstrated that interspecific triploid fish hybrids survive better than the corresponding diploid hybrids. Notable examples of this phenomenon include the tiger trout (brown trout × brook trout) hybrid, the rainbow trout × coho salmon hybrid, and the chum salmon × chinook salmon hybrid. The tiger trout has considerable potential as a sport fish and may be advantageous because both the diploid and triploid hybrids are essentially sterile. The rainbow trout × coho salmon hybrid has increased resistance to IHN (infectious hematopoietic necrosis) virus characteristic of the coho salmon parent. The chum salmon × chinook salmon hybrid has early seawater tolerance characteristic of the chum salmon parent.

Gynogenesis (all-maternal inheritance) experiments have normally involved complete inactivation of the paternal genome by radiation or chemical treatment of the sperm. However, we have demonstrated that if a lower than normal radiation treatment is applied to the sperm, some paternal genes may still be active in the progeny. This has been demonstrated for both pigmentation and isozyme loci. It appears that the paternal genes in this situation are located on chromosomal fragments which are lost during development. If the paternal genes can be stably inherited and if desirable paternal traits can be selected for, this "incomplete gyogenesis" might potentially be used to transfer desirable traits between species.

Androgenesis is induced by fertilizing radiation-inactivated eggs with normal sperm and by applying a pressure or heat treatment to block the first cleavage division and produce homozygous diploids. We have successfully induced androgenesis in rainbow trout and have also produced androgenetic progeny from homozygous androgenetic males. Androgenesis has a number of distinctive applications for aquaculture, including generation of homozygous clones and recovery of strains from cryogenically preserved sperm.

Outcrossed Lines of the Hard Clam *Mercenaria mercenaria*

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ABSTRACT

A large-scale breeding program has been initiated in South Carolina to achieve improved growth and survival of the hard clams, *M. mercenaria*. This interdisciplinary, multi-institutional program uses the facilities and personnel of the South Carolina Wildlife and Marine Resources Research Institute, the College of Charleston, the University of South Carolina, and Clemson University.

Nursery stocks of hard clams that had been selected for fast growth were obtained from Aquaculture Research Corporation ("ARC" - Dennis, MA) and the Virginia Institute of Marine Science ("VIMS" - Wachapreague, VA). These stocks were compared to corresponding wild populations for allele frequencies at seven polymorphic enzyme loci. Although as few as 30–60 parents were spawned at each of four generations to produce these two broodstocks, neither line exhibited any reduction in heterozygosity. Both lines, however, showed evidence of genetic drift and loss of rare alleles, suggesting that crosses between them could result in genetically distinct lines.

ARC and VIMS stocks were spawned on three occasions at different times of the year for production of both reciprocal outbred and pure control lines. Growth and survival were monitored regularly over two years. Early growth was strongly influenced by time of spawning, and as such was not a reliable indicator of subsequent growth. Most significant disparities between trials decreased as the lines aged. At 24 months, outbred and purebred lines were not consistently different in their heterozygosity, mean size, or size variance.

Within crosses, little relationship was detected between shell length and heterozygosity averaged over the seven enzyme loci. However, significant differences between the largest and smallest clams were detected at individual loci in 10 of 42 tests. Results were consistent neither with the hypothesis that the alleles themselves were affecting growth, nor with the hypothesis that these enzyme loci were tightly linked to other loci affecting growth. Rather, it appears that alleles are marking the entire genomes of their parents, and that variation in the growth rates of the offspring from individual clams may be obscuring any relationship with overall heterozygosity.

A Preliminary Study on Genetics of Two Types of the Rotifer *Brachionus plicatilis*

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ABSTRACT

The domesticated rotifer *Brachionus plicatilis* can be divided roughly into two types, called L and S, using morphological differences in the shape of anterior spines on the lorica (obtuse angled and pointed, respectively). However, differences in growth responses with respect to environmental factors make this method unreliable. We have, therefore, tried to clarify differences at the genetic level between types, using starch gel electrophoresis of enzymes.

Thirty-four collected strains were separated by three methods into the two types. Initially, strains were qualitatively judged with respect to differences in the shape of anterior spines. Afterwards pure strains were cultured parthenogenetically and re-evaluated using the second method (quantitative). To accomplish this, morphological features were measured, the ratios of which created an index for comparison of the strains (cluster analysis). Both the anterior spine and cluster analysis indicated that the 34 strains were composed of two large clusters consisting of 15 L and 19 S strains.

Allozyme variations of the 34 strains were then detected by horizontal starch gel electrophoresis. Nine isozyme loci were recognized. Of the 42 alleles observed, 15 alleles over 6 loci showed great differences between L- and S-types. Using genetic distances according to the allele frequencies of 42 alleles, a dendrogram was drawn. The strains separated into two groups. One group consisted of only S-type strains, the other group was subdivided again into 3 clusters. One of these three clusters consisted only of the S-type strains, while the other two contained only L-type strains. This result indicates the great genetic differences between L and S strains.

Introduction

Since the introduction of the rotifer *Brachionus plicatilis* to nourish larval fish, aquaculturists have increased scientific attention on this organism. In Japan a significant achievement in rotifer biology was the discovery that the domesticated rotifers can be divided roughly into two so-called S and L types as shown in Figure 1 (Fukusho 1983). The main morphological differences between the two types are lorica size, lorica shape, and the shape of the anterior spines on the lorica. They also exhibit differences in growth with respect to temperature. The morphological and physiological differences in the two types were summarized in a previous review (Hirayama 1987). The rotifer, especially the domesticated rotifer, exhibits cyclomorphosis (seasonal variation in size) and also polymorphosis (change in size influenced by variations in diet) (Fukusho and Iwamoto 1980, 1981). So, there is a probability that observed differences could be attributed to cyclo- or poly-morphosis, not to genetic differences. However, Fukusho and Okauchi (1982, 1983, 1984) have provided evidence that differences may be genetic and that the two types can be isolated from each other. In countries outside Japan, many scientists recognize the variation of rotifers which is due to polymorphosis. Scientific approaches concerning analysis of allozyme variation have therefore been investigated (Serra and Miracle 1983, 1985, 1987; Snell and Carrillo 1984; Snell and Winkler 1984; Suzuki 1983, 1987; King and Zhao 1987), while in Japan there have been no studies to detect allozyme variation in the two types by means of electrophoretic procedures.

Using strains collected from many locations, we attempted to distinguish L and S types using morphological comparisons. In order to confirm the genetic differences between strains, allozyme variations were detected by horizontal starch gel electrophoresis. Then, the genetic distances among collected strains were compared for morphological similarities.

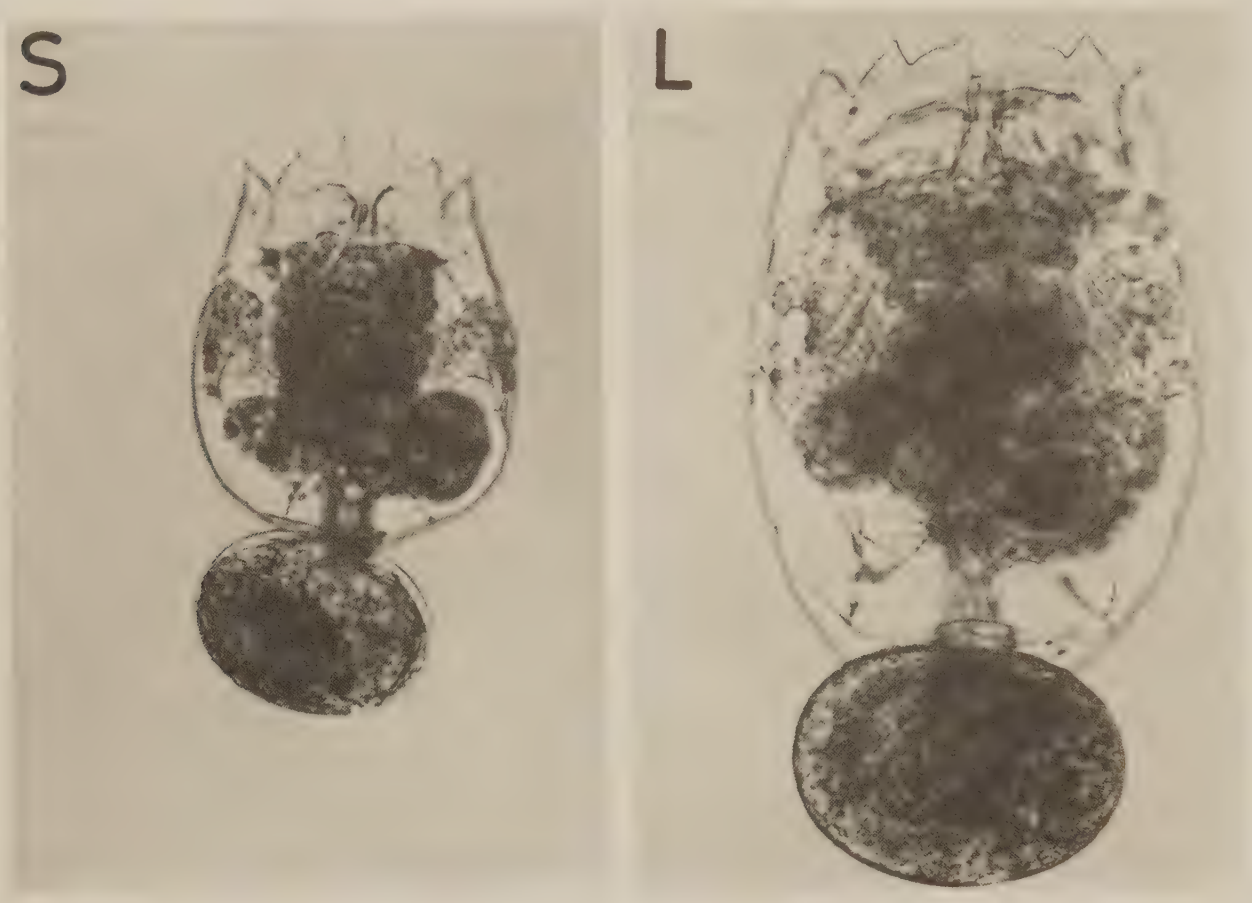

Figure 1
The two types of rotifer *Brachionus plicatilis*, L and S (provided by K. Fukusho).

Materials and Methods

We collected many strains from all over the world. On the map (Fig. 2), the localities of 34 strains used in this study are shown. Table 1 shows the abbreviated names and origins of the strains. In the tables and figures, L- and S-type strains are shown by abbreviation with capital and small letters, respectively.

Morphological Analysis

We first observed the anterior spines of each of the 34 strains and qualitatively divided them into the two types, L and S, according to whether they had obtuse angled or pointed spines, respectively. We classified 15 strains into the L type and 19 strains into the S type. After the initial screening, one individual from each strain was selected for culturing parthenogenetically and was regarded as one genetic strain for further study. Each strain was cultured with marine Chlorella (*Nannochloropsis oculata*). We collected eggs and recultured each strain in marine Chlorella suspensions in 23°C. The first eggs were laid after 48 hours. We collected those eggs into test tubes reculturing them again with marine Chlorella. After the offspring hatching from those eggs grew and laid their first eggs, we performed morphological measurements. We removed 20 individuals per sample and measured seven morphological features (Fig. 3, A through G). The ratios of these measurements were used to create indices for a cluster analysis.

Allozyme Analysis

The same 34 strains were used both for electrophoretic and morphological analysis. Allozyme analysis for each strain was conducted with a population grown from one individual and cultured with marine Chlorella and baker's yeast. The population was harvested with a net, washed with clean seawater several times, blotted dry using filter paper and frozen at -30°C until analyzed. Before harvesting, the group was starved for one day to remove the influences of food. Immediately prior to electrophoretic analysis, we thawed the sample and used a small amount of the drip absorbed by filter paper as a crude extract of enzyme for allozyme alayisis. Electrophoresis were carried

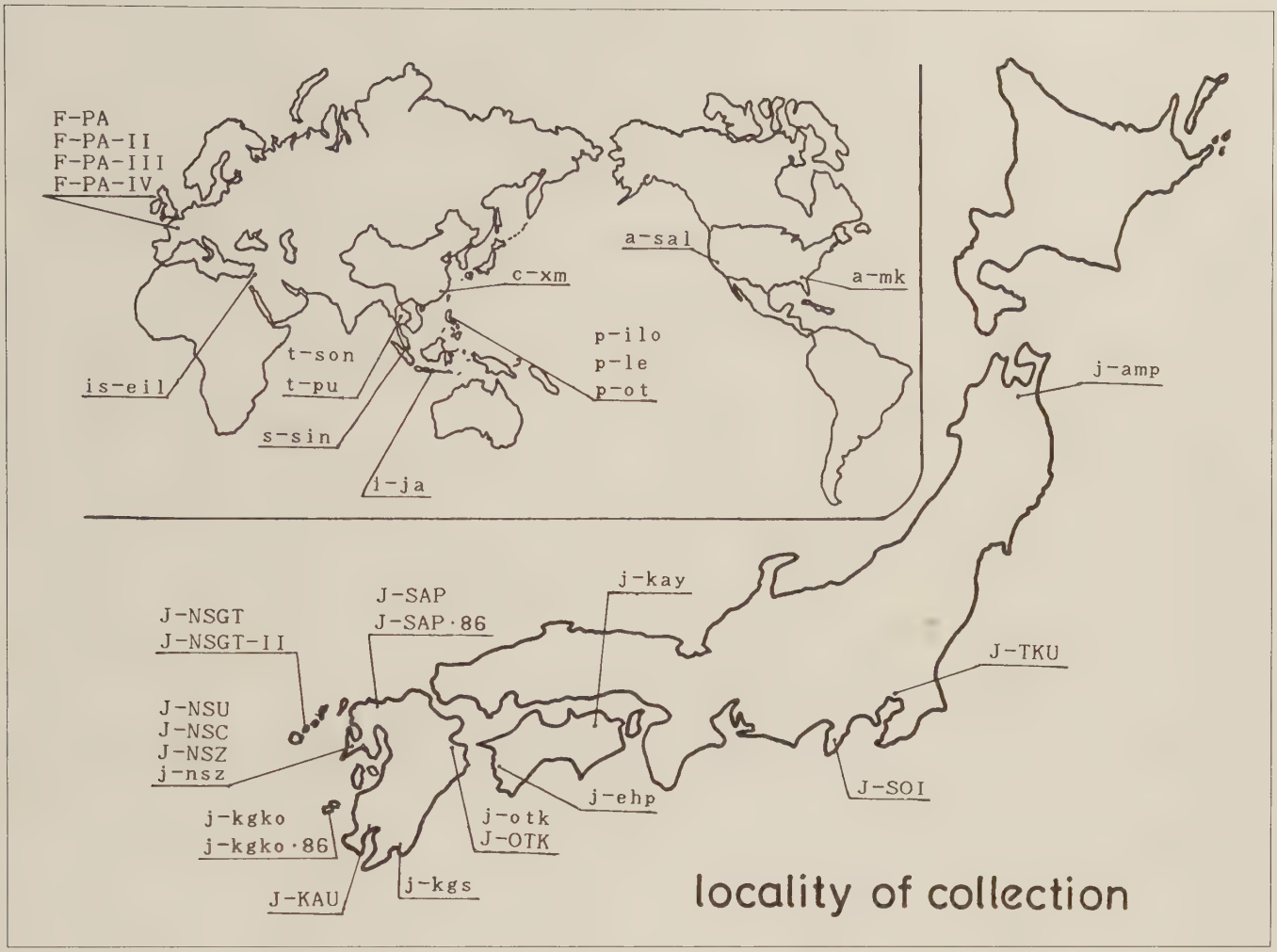

Figure 2
Map of collection localities. Capital and small letters mean L- and S-type strains, respectively.

out in 11% starch gel with three buffer systems reported by Clayton and Tretiak (1972) with minor modifications (Table 2). Staining procedures were from Shaw and Prasad (1970) and Siciliano and Shaw (1976). The following 18 enzymes were tested: α-Glycerophosphate dehydrogenase (αGPD, EC 1.1.1.8); D-Sorbitol dehydrogenase (SDH, EC 1.1.1.14); Lactate dehydrogenase (LDH, EC 1.1.1.27; 3-Hydrooxybutyrate dehydrogenase (HBDH, EC 1.1.1.30); Malate dehydrogenase (MDH, EC 1.1.1.37); Malic enzyme (ME, EC 1.1.1.40); Isocitrate dehydrogenase (IDH, EC 1.1.1.42); 6-Phosphogluconate dehydrogenase (6PGD, EC 1.1.1.44); Glucose-6-phosphate dehydrogenase (G6PD, EC 1.1.1.49); Superoxide dismutase (SOD, EC 1.15.1.1); Aspartate aminotransferase (AAT, EC 2.6.1.1); Adenylate kinase (AK, EC 2.7.4.3); Phosphoglucomutase (PGM, EC 2.7.5.1); Esterase (EST, EC 3.1.1.1); Alkaline phosphatase (ALP, EC 3.1.3.1); Acid phosphatase (ACP, EC 3.1.3.2); Leucine aminopeptidase (LAP, EC 3.4.11.1); and Glucose phosphaste isomerase (GPI, EC 5.3.1.9).

Results

Morphological Analysis

In Figure 3, are shown the average morphological measurements and standard deviations of the 15 L and 19 S strains. The results indicate that the strains of the rotifer could be divided clearly into the two types by quantitating the shape of the anterior spine (E/D, G/F). The results of the cluster analysis (Fig. 4A) are identical to the classification judging by the anterior spine shape (15 L types, 19 S types). Each cluster can be divided again into 2 small clusters. These results indicate that with statistical treatment of the morphologica charcteristics, the varieties of the

Table 1

Abbreviated names and origins of 34 strains of *Brachionus plicatilis* tested for morphological and genetic differences. PE: Prefectural Experimental Station or Hatchery; SFC: Japan Sea Farming Center; AQD SEAFDEC: Aquaculture Division of South East Asian Fisheries Development Center; NICA: National Institute of Coastal Aquaculture; and CE: City Hatchery. Capital and small letters mean that the strain belongs to L and S type respectively.

Abbreviated name	Country	Locality	Station or hatchery	Year of collection	Wild (w) or domesticated (d)
j-amp	Japan	Aomori	PE	'87	d
j-kay	Japan	Kagawa	PE	'87	d
j-ehp	Japan	Ehime	PE	'87	d
j-otk	Japan	Oita	SFC	'87	d
j-nsz	Japan	Nagasaki	PE	'86	d
j-kgko	Japan	Kagoshima (Kai Lake)		'78	w
j-kgko·86	Japan	Kagoshima (Kai Lake)		'86	w
j-kgs	Japan	Kagoshima (Shibushi	SFC	'87	d
a-sal	USA	California (Salton Sea)		'78	w
a-mk	USA	Florida (Makay Bay)		'80	w
c-xm	China	Fujian Fish. Res. Inst.		'87	d
p-ilo	Philippines	Panay Island	AQD SEAFDEC	'84	d
p-le	Philippines	Panay Island	Leganes Stn. AQD SEAFDEC	'84	d
p-ot	Philippines	Oton River (Panay Island)		'84	w
i-ja	Indonesia	Java		'86	d
s-sin	Singapore	Natl. Inst. of Aquaculture		'86	d
t-son	Thailand	Sonkia	NICA	'87	d
t-pu	Thailand	Puket Marine Inst.		'87	d
is-eil	Israel	Eilat		'87	d
J-SOI	Japan	Shizuoka	PE	'78	d
J-TKU	Japan	Univ. Tokyo		'78	d
J-OTK	Japan	Oita (kamiura)	SFC	'87	d
J-SAP	Japan	Saga	PE	'84	d
J-SAP·86	Japan	Saga	PE	'86	d
J-NSU	Japan	Nagasaki Univ.		'69	d
J-NSZ	Japan	Nagasaki	PE	'86	d
J-NSC	Japan	Nagasaki	CE	'86	d
J-NSGT	Japan	Nagasaki (Goto Island)	SFC	'87	d
J-NSGT-II	Japan	Nagasaki (Goto Island)	SFC	'87	d
J-KAU	Japan	Kagoshima Univ.		'86	d
F-PA	France	Palavas-les-Flots		'87	d
F-PA-II	France	Palavas-les-Flots		'87	d
F-PA-III	France	Palavas-les-Flots		'87	d
F-PA-IV	France	Palavas-les-Flots		'87	d

Table 2

Buffer systems used for electrophoresis of enzymes.

Abbreviated name	Electrode buffer		Gel buffer		References
	Components	pH	Components	pH	
C-A	0.04 m Citric acid, adjust pH up to 6.1 with N-(3-aminopropyl)-morpholine.	6.1	Dilute 50 mL of electrode buffer to 1 liter (Citric acid, 0.002 M).	6.1	Clayton and Tretiak (1972)
C-A	0.04 m Citric acid, adjust pH up to 6.1 with N-(3-aminopropyl)-morpholine, then to 6.9 with NaOH.	6.9	Dilute 50 mL of electrode buffer to 1 liter (Citric acid, 0.002 M).	6.9	Clay and Tretiak (1972)
C-T	0.04 m Citric acid, adjust pH up to 8.0 with Tris-(hydroxymethyl)-methylamine.	8.0	Dilute 50 mL of electrode buffer to 1 liter (Citric acid, 0.002 M).	8.0	Clayton and Tretiak (1972)

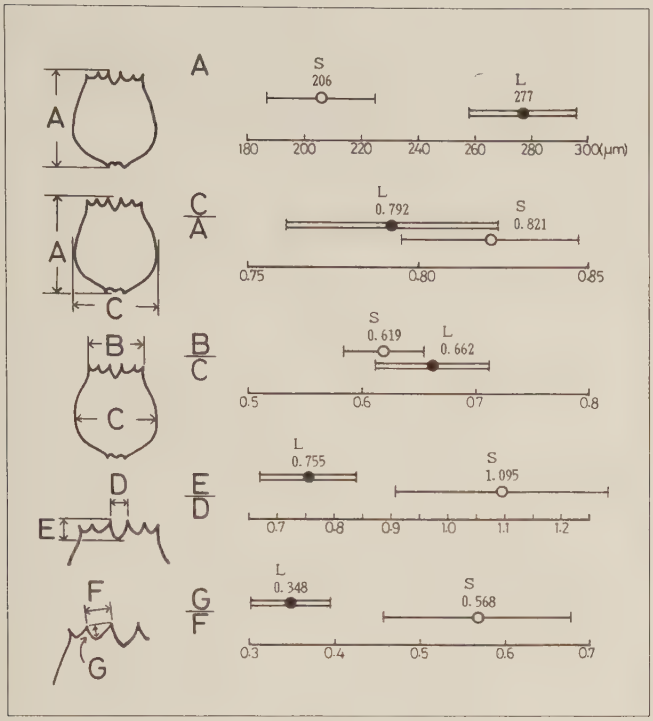

Figure 3
Averages and standard deviations of 5 varieties of measurements considered for differentiating L- and S-type strains.

rotifer can be divided into two groups, and that the strains within the same type display further variation.

Allozyme Analysis

Among 18 enzymes tested, 10 enzymes showed clear banding patterns (Table 3). However, bandings for 3 enzymes (AK, EST, and IDH) were not genetically interpretable. The number of alleles of each locus are summarized in Table 4. On MDH, 3 isozyme loci were recognized, although no alleles were detected at 2 loci. In Table 5 are shown the number of L- and S-type strains and the alleles they posses at each locus. The L and S strains differ considerably in allele profiles. For instance, at *Ldh* where 8 alleles were observed, 9 of 15 L strains possessed the A allele whereas none of S strains possessed the A allele. In contrast, B allele appeared only in the S strains. There were considerable genetic differences between L and S strains for 15 alleles at 6 loci. Allele frequencies for each allele at 9 loci affecting 7 enzymes were estimated for each strain in which individuals were considered to be genetically identical. For MDH, however, three zones of banding patterns appeared. Although two of those three zones were not interpretable as showing allozyme variation, we regarded allele frequency as one if the strain had the bandings in

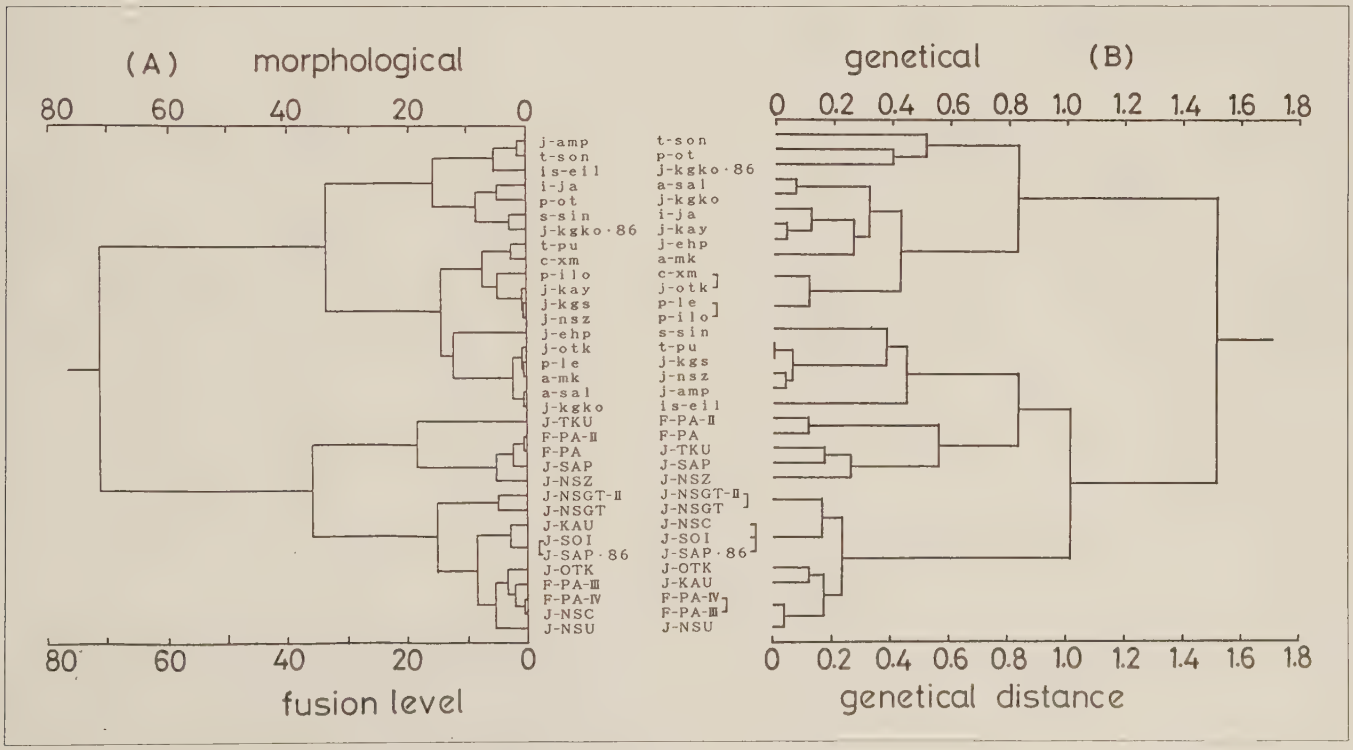

Figure 4
Dendrograms of similarities among 34 strains by morphological and genetic snalyses. Abbreviated names by capital and small letters mean that the strain belongs to L and S type, respectively.

Table 3

The different enzyme systems of *Brachionus plicatilis* screened with various buffers. × = no detectable bandings; ▲ = unclear bandings; ● = find bandings.

Enzyme	Buffer C-A (pH 6.1)	C-A (pH 6.9)	C-T (pH 8.0)
αGPD	×	×	×
SDH	×	×	×
LDH	▲	●	▲
HBDH	×	×	×
MDH	▲	●	▲
ME	×	▲	▲
IDH	▲	▲	●
6PGD	▲	●	×
G6PD	×	▲	×
SOD	●	●	▲
AAT	▲	●	▲
AK	●	●	▲
PGM	×	●	▲
EST	▲	●	▲
ALP	▲	▲	▲
ACP	▲	▲	▲
LAP	×	×	×
GPI	▲	▲	●

Table 4

Isozyme loci and number of observed alleles.

Enzyme	Locus	Allele	Subunit structure
LDH	*Ldh*	8	Tetramer
MDH	*Mdh-I*	3	Dimer
	Mdh-II	?	—
	Mdh-III	?	—
6PGD	*6Pgd*	9	Dimer
SOD	*Sod*	4	Dimer
AAT	*Aat-I*	2	Dimer
PGM	*Pgm-I*	8	Monomer
GPI	*Gpi*	6	Dimer

Table 5

The number of L- and S-type strains for each allele at different enzyme loci. ● = Great difference in allele possession between L- and S-type strains.

Locus	Allele	Relative mobility (%)	L-Type (15)	S-Type (19)
Ldh	X	100	2	0
	● A	81	9	0
	● B	69	0	5
	C	64	4	4
	● D	47	1	12
	E	39	1	3
	O_1	—	0	2
	O_2	—	1	0
Mdh-I	A	100	0	3
	B	83	13	16
	C	62	15	9
Mdh-II	● ?	—	0	17
Mdh-III	● ?	—	15	6
6Pgd	A	100	0	1
	● B	91	10	0
	B^L	75	1	1
	C	68	4	1
	C^L	55	2	0
	● D	(−)39	0	10
	D^L	(−)52	0	1
	● E	(−)68	0	13
	F	(−)75	0	1
Sod	● A	100	0	6
	● B	95	10	0
	● C	74	0	17
	● D	49	5	0
Aat-I	A	100	11	8
	B	75	15	15
Pgm-I	A	100	4	6
	B	93	3	2
	C	89	1	2
	D	86	7	6
	E	79	3	9
	F	77	0	1
	G	70	0	2
	O	—	1	0
Gpi	● A	100	0	6
	B	78	0	1
	C	67	2	6
	● D	43	0	13
	● E	28	13	0
	F	0	7	2

the zone. If not, we decided allele frequency on the zone as zero. According to Nei's formula (1972), the genetic distances among the 34 strains were estimated from gene frequencies including estimated values for MDH. The dendrogram expressing similarities among the 34 strains was also drawn from genetic distances (Fig. 4B). The 34 strains can therefore be divided into two major groups. One group consists only of the strains which had been identified as S type judging by the anterior spine shape. The other cluster can be divided again into 3 smaller clusters, one of which consists only of S type strains, and the other two clusters consist only of L-type strains. Although one of the two large clusters includes the two types of rotifers, the classification by the genetic distances also pointed out that there are great genetical distances between L and S strains. Some of the strains which are genetically identical (e.g., genetic distance = 0) were collected from neighboring loca-

tions or from the same hatchery, for instances between the two strains of p-ilo and p-le or between J-NSGT and J-NSGT-II. However, in one instance (c-xm and j-otk), the samples were geographically unrelated.

Discussion

For comparison, the two dendrograms are shown in the same frame (Fig. 4). The dendrogram patterns for the two methods are very similar, especially with respect to the L-type strains.

The results indicate that the rotifer *Brachionus plicatilis* can be divided into the two types of genetic constitution.

The results in this report were drawn from 34 strains, collected mainly from western Japan. In the case of the L-type, the overseas strains obtained came from only one locality. We are now collecting more strains from all over the world in order to make a more unequivocal conclusion.

Acknowledgments

The authors wish to express their sincere thanks to H. Kayano, Nagasaki University, for his kind advice on the interpretation of allozyme variation, to K. Fukusho who kindly provided photos of L and S strains, and also to the scientists who kindly sent us live samples of the rotifers.

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Present Status of Genetic Studies on Marine Finfish in Japan

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ABSTRACT

The present paper briefly introduces the status of genetic breeding of marine finfish in Japan. The domestication of exotic species is also described. Selection is the most successful technique of genetic breeding for marine finfish, even though limited scientific data and experimental results have been reported. Selection was conducted on red sea bream, *Pagrus major*, and Japanese flounder, *Paralichthys olivaceus*, and their selected strains were supplied for industrial culture. Experiments on hybridization leading to heterosis were conducted as well as interspecific, intergeneric, interfamily, back and reciprocal crosses. Few of these hybrids, however, have been widely used by the industry, except the *P. major* × crimson sea bream, *Evynnis japonicus*. Chromosome manipulation studies such as triploid production and all female production by gynogenesis have been conducted since 1984 in Japan. These technologies are strongly expected to be adopted in industrial culture, even though they are currently experimental. Exotic species or strains of marine finfish have been introduced to Japan and cultured in recent years. Most marine species are imported to supply seed where local production is inadequate, not to introduce a new industrial target species or strain, except the red sea bream. Cryopreservation of sperm is used in most hybridization studies, induction of gynogenesis, and triploid production. This technology will no doubt be adopted in androgenesis and gene bank projects for marine finfish.

Introduction

Mariculture of finfish is well developed in Japan as reflected by the total harvest in 1986 of nearly 2×10^5 tons. The number of cultured species is approximately 30 (Table 1) (Fukusho 1981). Yellowtail, *Seriola quinqueradiata* (14.6×10^4 tons), and red sea bream (3.4×10^4 tons), are the most important species. The Japanese flounder and coho salmon, *Oncorhynchus kisutch*, have shown great promise as cultured species, with their production levels increasing rapidly in recent years (0.3×10^4 and 1.2×10^4 tons, respectively for 1987 data). All the marine species are cultured in net cages except the Japanese flounder which is usually raised in land based tanks.

At present, the total supply is adequate to satisfy demand for yellowtail and red sea bream, taking into consideration the total comsumptiom plus the carrying capacity of the culture ground. Therefore, research presently focuses on the improvement of fish quality (e.g., high growth rate, high resistance to pollution, good taste and flavor, and favorable color) based on the requirements of culturists and consumers. Genetic breeding is one of the most effective tools for improving fish quality. Therefore, various kinds of experiments on genetics, including chromosome manipulation have been intensively conducted in recent years for marine finfish, despite the short history of mariculture.

The objective of the present paper is to provide a brief introduction on the status of genetic breeding of marine finfish, except salmonids in Japan. Introductions leading to domestication are considered part of the study of genetics for the purpose of this review.

The Introduction of Exotic Species

Exotic species or strains of marine finfish, such as yellowtail, red sea bream, rockfish, *Sebasticus* spp., grouper *Epinephelus* spp., knifejaw *Oplegnathus* spp., have been introduced to Japan and cultured in recent years. Marine finfish have been introduced for different reasons than have freshwater fish. Most marine species are imported to supply seed where the local production is inadequate, not to introduce a new industrial target species of strain. An exception is the case of the red sea bream.

Table 1
Marine finfish cultured in Japan (Fukusho 1981).

Family	Common name	Scientific name	Pref. No.[a]
Salmonidae	Coho salmon	*Oncorhynchus kisutch*	1
Mugilidae	Grey mullet	*Mugil cephalus*	1
Oplegnathidae	Japanese striped knifejaw	*Oplegnathus fasciatus*	19
	Japanese spotted knifejaw	*O. punctatus*	7
Serranidae	Sea bass	*Lateolabrax japonicus*	5
	Sea bass	*L. latus*	1
	Groupers	*Epinephelus* spp.	3
Girellidae	Nibbler	*Girella punctata*	11
Sparidae	Red sea bream	*Pagrus major*	25
	Porgy	*Sparus sarba*	2
	Crimson sea bream	*Evynnis japonica*	11
	Porgy	*Acanthopagrus schlegeli*	17
	Porgy	*A. latus*	2
Pomadasyidae	Grunt	*Parapristipoma trilinea*	1
Carrangidae	Yellowtail	*Seriola quinqueradiata*	26
	Amberjack	*S. purpurascens*	8
	Amberjack	*S. aureovittata*	2
	Horse mackerel	*Trachurus japonicus*	20
	Striped jack	*Longirostrum delicatissimus*	9
Scombridae	Bluefin tuna	*Thunnus thynnus*	3
Siganidae	Rabbit fish	*Siganus fuscessens*	3
Aluteridae	Filefish	*Stephanolepis cirrhifer*	2
Tetradontidae	Puffer	*Fugu rubripes*	9
Scorpaenidae	Rockfish	*Sebastiscus marmoratus*	3
	Rockfish	*Sebastes inermis*	
	Rockfish	*S. schlegeli*	1
Bothidae	Flounder	*Paralichthys olivaceus*	10

[a]Number of prefectures where the species was cultured.

A deep reddish color is highly prized in cultured red sea bream by the Japanese consumers. A Korean strain, which is identical to the Japanese strain taxonomically, shows a much deeper reddish color than the latter (Harada et al. 1988, Harada et al. 1985, Kumai et al. 1986). No difference has been found between the two strains in electrophoresis analysis of isozymes. The Korean strain is preferred by fish farmers because of its deeper reddish color, even though the Japanese strain is superior to the Korean strain in terms of growth rate (Kumai et al. 1986). The deep reddish color is caused by its higher content of carotenoid and astaxanthine in the skin, which results even under the same rearing and feeding conditions as the Japanese strain (Kumai et al. 1986). Hybridization between the Japanese and Korean strains has been conducted to provide a hybrid with a deeper reddish color and higher growth rate (Harada et al. 1988).

Both fertilized eggs and juveniles of Japanese red sea bream have been exported to foreign countries; Thus marine finfish have been introduced world-wide and mariculture has expanded. In each country, they are being cultured as an introduced species. Precise investigation and research of a new marine species' biological characteristics and response to new environmental conditions should always be considered prior to introduction, as with freshwater fish.

Hybridization

Experiments on hybridization leading to heterosis have contributed to the development of larval rearing techniques for marine finfish (Fujita 1961, 1967; Harada 1974, 1975, 1978). Interspecific, intergeneric, interfamily, back and reciprocal crosses have also been attempted (Harada 1978).

Hybridization of marine finfish was initiated on puffers (1961–67) of which several species are a high prized luxury food despite the fact that parts of these fish are highly toxic (Fujita 1967) (Table 2). The Fisheries Laboratory of Kinki University has further promoted hybridization to improve fish quality. Several successful and promising hybrids were produced that are superior in growth rate, survival rate, body color, and meat quality to each parent fish (Table 3) (Harada 1974, 1975, 1978). The "kindai" (*Oplegnathus fasciatus* × *O. punctatus*), which abbreviates the name of Kinki University and which means sea bream and sounds like "golden fish" in Japanese, is significantly superior in growth rate, survival rate, and handling to each

Table 2
Hybridization of puffers (Fujita 1967).

Female		Male	Hatching rate (%)
Takifugu pardalis	×	*T. poecilonotus*	95
T. poecilonotus	×	*T. pardalis*	95
T. rubripes	×	*T. xanthopterus*	93
Lagocephalus lunaris spadiceus	×	*T. niphobles*	0
L. l. spadiceus	×	*Canthigaster rivulata*	0

parent fish (Kumai 1984; Harada et al. 1986). Because red sea bream are usually cultured in protected bays with varying salinity, tolerance to low salinity is an important characteristic. Therefore, a useful hybrid of the red sea bream, and the porgy, *Acanthopagrus shulegeli*, was developed which showed both improved tolerance to lower salinity than the maternal fish plus faster growth and better taste than the paternal one (Harada 1975). The Nagasaki Prefectural Institute of Fisheries has also conducted studies on the hybridization of marine finfish. A hybrid of a sparid, *Sparus sarba*, and the porgy, *A. shulegeli*, was produced in order to combine the highest growth characteristics of *S. sarba* and the low salinity resistance of *A. schulegeli* (Kitajima and Tsukashima 1983). However, the hybrid showed maternal characteristics in both its morphological and physiological characteristics (Kitajima and Tsukashima 1983). The same phenomenon occurred in the hybrid of *P. major* and the crimson sea bream, *Evynnis japonicus* (Arakawa and Yoshida 1986, Arakawa et al. 1988).

Few of these hybrids have been widely used by industry, except the hybrid of *P. major* × *E. japonicus*. The reason may be due to 1) conservative consumers to whom appearance is very important (red sea bream must look like the wild red sea bream because the Japanese people always eat the whole and raw fish for sashimi and sushi; 2) limited attempts to show clearly the difference in quality between the hybrid and parent fish; and 3) too short a period of marine finfish culture for the industry to evaluate and utilize new strains or hybrids as well as exotic species.

Selection

Selection is the most successful technique of genetic breeding for marine finfish, even though limited scientific data and experimental results have been reported. Selected strains of red sea bream have been supplied by the Fisheries Laboratory of Kinki University. These selections are highly desired by fish farmers because their growth rates are approximately 30–40% higher than the wild forms. Mass selection has been conducted at the Fisheries Laboratory of Kinki University over several generations from wild red sea bream caught in the Akashi area in the Seto Inland Sea. Production of selected strains has also been intensively conducted on the Japanese flounder and the Japanese striped knifejaw, *O. fasciatus*, at the Fisheries Laboratory of Kinki University (Harada 1975, 1978).

Table 3
Hybrids of marine finfish produced at the Kinki University (Harada 1974, 1975, 1978).

Female		Male	
Pagrus major	×	*Acanthopagrus schulegeli*	1964
P. major	×	*Sparus sarba*	67
Oplegnathus fasciatus	×	*A. schlegeli*	68
O. fasciatus[a]	×	*O. punctatus*	69
Seriola quinqueradiata[a]	×	*S. auroeovittata*	70
S. quinqueradiata	×	*S. purpurascens*	70
S. purpurascens[a]	×	*S. aureovittata*	71
S. aureovittata	×	*S. purpurascens*	72
P. major[a]	×	*Evynnis japonicus*	73
O. puntatus[a]	×	*O. fasciatus*	73
Auxis thazard	×	*Euthynnus affinis*	76

[a]Promising hybrid.

Chromosome Manipulation

Since 1984, chromosome manipulation studies such as triploid production, all female production by gynogenesis, extraction and synthesis of growth hormone, production of cloned fish, and cell fusion have been conducted in Japan to produce new strains of marine finfish. These technologies have been called "Fisheries Biotechnology." In 1985, the Ministry of Agriculture, Forestry, and Fisheries (MAFF) designed and organized a large-scale scientific project on chromosome manipulation titled "Development of new breeding techniques by induction of gynogenesis in fish." The National Research Institute of Aquaculture was the leading institution to promote the project, along with three Universities (Tokyo University of Fisheries, Hokkaido University, and Kansei Gakuin University), two Regional Fisheries Laboratories (Nansei Regional Fisheries Laboratory and Hokkaido Regional Fisheries Laboratory of the Fisheries Agency-MAFF), and three Prefectural Institutes of Fisheries (Saitama, Hyogo, and Hokkaido). The target species included the Japanese flounder, red sea bream, plaices, *Verasper moseri*, *Limanda shrenki*, *L. punctatissima*, *Platichthys stellatus*, and filefish, *Stephanolepis cirrhifer*, *Thamnaconus modestus*, *Aluterus monoceros*. The Fisheries Agency has also organized and initiated a scientific project addressing fisheries biotechnology. Several Prefectural fisheries laboratories have also joined this "Local Biotechnological Study Project" where marine

finfish such as the red sea bream and flounder are being studied.

Triploid and gynogenetic diploid induction techniques which use cold shock have been used to block the second polar body extrusion for red sea bream (Arakawa et al. 1987; Arakawa and Miyahara 1988; Fukusho et al. 1987b), porgy (Arakawa et al. 1987), and flounder (Tabata, 1988; Tabata and Gorie 1988a, Tabata et al. 1986). The duration of cold shock intervals are as follows: for red sea bream, 15–20 min. duration of 0°C, starting 3 min. after insemination; for porgy, 25 min. duration, starting 1.5 min. after insemination; and for flounder, 45 min. duration, starting 3–5 min. after insemination. Suppression of the 1st cleavage was achieved by using increased hydrostatic pressure (Tabata and Gorie 1988b). UV irradiation has been effective for genetic inactivation of sperm (e.g., 1000–2000 erg/mm^2 for red sea bream). Also, sperm of different species have often been used as an indicator of the induction of gynogenesis (Fukusho et al. 1987a; Yano and Sakai 1988). Triploids were produced in red sea bream and porgy (Arakawa et al. 1987, 1988; Fukusho et al. 1987b). Thus, various conditions for induction of triploid and gynogenesis have been examined for several marine finfish, and comparisons of growth rate, survival rate, and other biological characteristics have been conducted through larval rearing (Arakawa and Yoshida 1986; Fukusho et al. 1987a; Tabata et al. 1986; Tabata and Gorie 1988a). Comparison of growth rate during rearing to young stage and commercial size was also conducted (Tabata and Gorie 1988a), but there is little information to evaluate gynogenetic and triploid fish in view of industrial culture because the scientific activities have only just started. Reports and papers on the comparison of growth and maturation rates and sex ratio between chromosome manipulated fish and common diploid fish are expected to promote these techniques in industrial mariculture of finfish. Techniques for examination of ploidy have been established, but with conflicting result (e.g., appearance of males among gynogenetic diploids of the flounder in spite of a theoretical expectation of all female production) (Tabata 1988; Tabata and Gorie 1988b). These phenomena could not be explained by the XX and XY sex chromosome theory. Further investigation on embryology and sexual differentiation might be required as well as genetic studies. The mechanism of sexual differentiation should be clarified to advance the technology of chromosome manipulation.

Cryopreservation of Sperm

Experiments on androgenesis have been conducted for freshwater fish in Japan. Cryopreservation of sperm in combination with androgenesis is useful in preserving endangered species, and also in all male production. Cryopreservation is also used in most hybridization studies, induction of gynogenesis, and triploid production.

In marine fish, cryopreservation has been conducted on a variety of species, such as: two species of goby, *Glossogobius olivaceus*, *Acanthogobius flavimanus*; porgy; mullet, *Mugil cephalus*; mackerel, *Scomber japonicus*; bluefin tuna, *Thunnus thynnus*; and puffer, *Takaifugu nipholbles* (Doi et al. 1982; Kurokura 1983). A recent study on the hybridization of red sea bream and crimson sea bream showed positive results with high survival rates and increased growth rates observed when sperm preserved for 6 months was used (Kurokura et al. 1986).

Thus, gamete preservation is useful for hybridization of species which spawn in different seasons, genetic breeding by chromosome manipulation, transplantation (introduction), and gene bank projects for marine finfish.

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Recombinant Viral Vaccines in Aquaculture*

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ABSTRACT

Viral pathogens in aquaculture have largely been controlled by the culling and destruction of carriers and infected animals and eggs. Because most viral pathogens in aquaculture are transmitted via water and because sensitive animals reside in the neighboring waters, the administration of attenuated viral vaccines has not been feasible. Attenuated vaccines require costly trials to assure that these modified live viruses are nonvirulent in all species and that reversion to virulence does not occur. Killed viral vaccines have been too expensive to produce for the aquaculture industry. Thus, subunit viral vaccines developed by recombinant DNA techniques are attractive alternatives for the industry. These vaccines are nonreplicating and inexpensive to produce. The molecular cloning and expression of viral genes in several host vector systems for the development of subunit viral vaccines for aquaculture has been the primary research focus of the authors' laboratory. Work on the development of such vaccines for infectious hematopoietic necrosis virus (IHNV), a fish rhabdovirus, and infectious pancreatic necrosis virus (IPNV), a fish birnavirus, is presented. Laboratory tests of both vaccines *in vivo* have indicated that fish develop protective immunity to live virus after vaccination.

Introduction

One of the major factors that will have an impact on the success of the aquaculture industry is the control of diseases. As the industry grows and greater productivity demands are made on facilities, the incidence of disease outbreaks will increase. Thus, the need for more effective disease controls has been receiving more attention. The viral diseases are particularly important because there are no suitable treatments available. In the United States, there are no approved antiviral drugs or vaccines that can be used in the aquaculture industry today.

At the present time, the control of viral diseases is based largely on management. Current recommendations for the control of viral disease outbreak include the destruction of diseased stocks, drainage of ponds, disinfection of contaminated areas with chlorine, treatment with sunlight or lime, and the restocking of the facility with disease-free stock. These procedures are very expensive and instituted with understandable reluctance by the industry. With earthen ponds and stable viruses, like the baculoviruses and the picornaviruses, these disinfection procedures may not work.

Another facet of present day controls for viral diseases in aquaculture is the requirement for certified pathogen-free stocks and eggs and the use of specific pathogen-free water in the facility. When available, these requirements have been very effective in preventing disease outbreaks. However, it has not always been possible to obtain disease-free stocks for highly-prized strains nor economically practical to use specific pathogen-free water. Thus, the aquaculture industry has a definite need for viral vaccines. Our group reports here the successful development of two prototype viral vaccines by recombinant DNA techniques.

Two viruses were selected for vaccine development because these viruses affect economically important aquaculture species (salmon and trout) in the United States, Europe, and Japan. In addition, these viruses, infectious hematopoetic necrosis virus (IHNV), and infectious pancreatic necrosis virus (IPNV), affect very young fish, and immunization of large numbers of fish at this size by immersion is fairly easy. IHNV is a rhabdovirus with an enveloped virion and glycoprotein peplomers on the

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envelope surface; it has a viral genome of single-stranded RNA of negative sense (McAllister and Wagner 1977). In contrast, IPNV is a nonenveloped virus with a single capsid and a genome of two segments of double-stranded RNA (Dobos 1975). The techniques that were involved in the construction of recombinant plasmids containing the genes for the major immunogenic proteins of these two viruses have been described (Kurath and Leong 1985; Huang et al. 1986). The expression of these proteins in bacteria and the use of these expressed proteins as vaccines will be described here.

Materials and Methods

Cells, Virus, and Antisera

The following viruses here used in this study: the IHNV isolate from Round Butte was obtained from W. Groberg (Oregon Department of Fisheries and Wildlife) and the IPNV isolates, Sp and Buhl, were obtained from R. Hedrick, University of California at Davis. The virus used for challenge studies was prepared by infecting rainbow trout (*Oncorhynchus mykiss*) fry and reisolating the virus from fish dying of IHN disease in the case of the IHNV isolates and IPN disease in the case of the IPNV isolates. Subsequently, the virus was grown for two passages in chinook salmon embryo cells (CHSE-214 cells) (Fryer et al. 1978). The tissue-culture supernatant fluid containing the virus was used as the challenge virus. The IHNV and IPNV used as molecular weight markers in Figure 1 were prepared as described in Kurath and Leong (1985) for IHNV and Huang et al. (1986) for IPNV. The rabbit antisera prepared against purified IHNV and IPNV were made as described (Engelking and Leong 1989).

Construction of Recombinant Plasmids

The construction of a recombinant plasmid containing the *trp* E promoter and the *trp* E gene fused to an immunogenic region of the gene for IHNV glycoprotein gene or the IPNV VP2 gene is shown schematically in Figure 2. The isolation, cloning and sequence analysis of these genes have been reported (Koener et al. 1987; D. S. Manning 1988). The pATH vectors were the generous gift of A. Tzagaloff (Dieckmann and Tzagoloff 1985). The constructions were verified by DNA sequence analysis by the dideoxy method (Sanger et al. 1977). The plasmid pUC 19, which served as the negative control for pTA1 in Figure 1 was obtained from Pharmacia, Inc., Piscataway, NJ.

Immunization Trials in Fish

Bacterial crude lysates were prepared as described (Kleid et al. 1981). Proteins were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western immunoblotting as previously described (Gilmore et al. 1988). The crude lysates were used to immunize fish by immersion. Rainbow trout fry at 0.4 g were immunized in sets of 100 fish. Immunization was accomplished by bathing groups of 100 fry in 25 mL of the vaccine preparation (ca. 3 mg/mL total protein concentration) for 1 minute. At that time, the immersion solution volume was increased to 250 mL with water and fish were incubated in this diluted solution for an additional

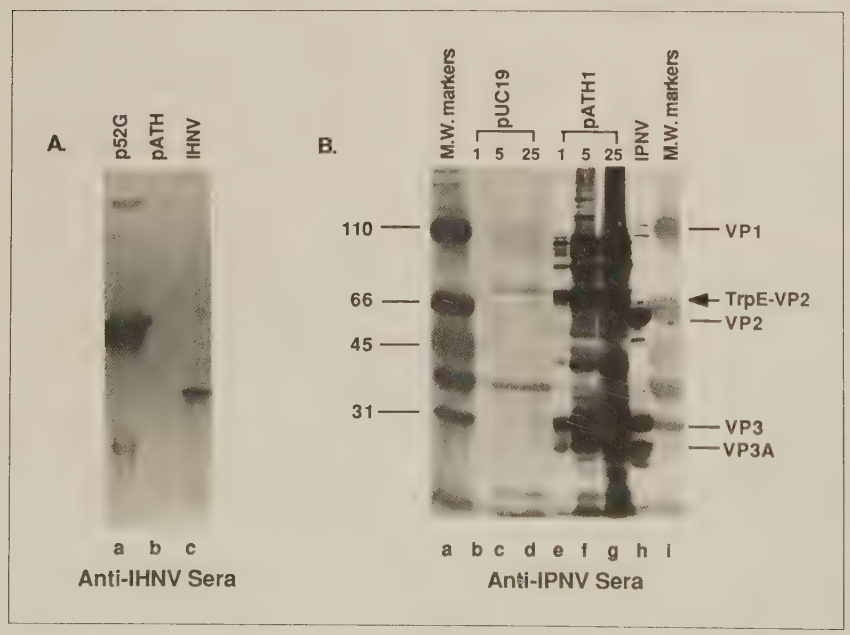

Figure 1

Analysis of bacterial production of *trp* E-viral gene fusion proteins by antibody reactivity on an electrophorectic transfer blot of a 10% SDS-polyacrylamide gel of bacterial extracts. (**A**) Development of the blot made with anti-IHNV sera. Lane a is the *trp* E-G fusion protein detected in cells containing the recombinant plasmid, p52G; Lane b are proteins detected in cells containing the expression vector pATH3, without a viral gene insert; and Lane c is purified IHNV. (**B**) Development of the blot made with anti-IPNV sera. Lanes a and i contain the molecular weight marker proteins: phosphorylase B (110000 Da); bovine serum albumin (66000 Da); ovalbumin (45000 Da); and carbonic anhydrase (31000 Da). In lanes b, c, and d are cell lysates from bacterial cells containing the plasmid pUC19 with no viral insert; the samples were loaded at 1, 5 and 25 μL respectively in lanes b, c, and d. In lanes e,

2 minutes. These fish were then placed in aquaria of 5 gallons with a water flow rate of 0.25 gal/min in a constant water temperature of 10°C. The control fish were exposed to saline in the same procedure or left undisturbed.

Approximately one month after immunization, the experimental and control fish were placed in separate aquaria in groups of 25. The fish were exposed to serial log virus dilutions in 1 liter of water. The challenge virus was prepared as described by Engelking and Leong (1981). In Figure 3, the data for fish exposed to $7.2 \times 10^{+5}$ plaque forming units per mL (PFU/mL) is presented. The data represents the mean of duplicate experiments. All dead fish were assayed for the presence of infectious virus in chinook salmon embryo cells (CHSE-214) as described by Engelking and Leong (1981).

Results

Antigen Production in Bacteria

The size and quantity of virus-specific antigen produced in bacteria hosting the recombinant plasmids was estimated from stained gels and Western blots of total bacterial extract. In Figure 1, the product of a *trp*E-IHNV glycoprotein fusion gene from the plasmid p52G and the major capsid protein of IPNV from the plasmid pTA1 is shown in Western blots of the appropriate bacterial lysates. A determination of the DNA sequence of p52G indicated that a 264 bp fragment of the IHNV glycoprotein gene had been inserted in-frame with the *trp*E protein to produce a fusion protein of 49000 daltons (49 kDa = 37 kDa [*trp*E] + 11 kDa [glycoprotein gene fragment]). In addition, a second fragment of the IHNV glycoprotein gene had been inserted out-of-frame adjacent to the 264 bp fragment and this additional nucleic acid resulted in 1 kDa (84 bp extra) more of amino acid residue owing to the fusion protein (Gilmore et al. 1988).

The IPNV expressing plasmid, pTA1, contained the entire coding region of the A segment of the viral genome for the isolate Sp. It was constructed so that the viral genetic information was fused in-frame to the *trp*E protein (Figure 2) and all the proteins encoded by the A segment were synthesized in the bacteria. Thus, VP2 (major capsid protein), and VP3 (minor capsid protein)

Figure 1 (Continued)
f, and g are 1, 5 and 25 μL of cell lysates from bacterial cells containing the plasmid pTA1. Lane h contains purified IPNV. The arrow indicates the *trp*E-VP2 fusion protein found in lanes e, f, and g. The symbol VP1 indicates virion protein 1; VP2, virion protein 2; VP3, virion protein 3; and VP3a, breakdown product of VP3.

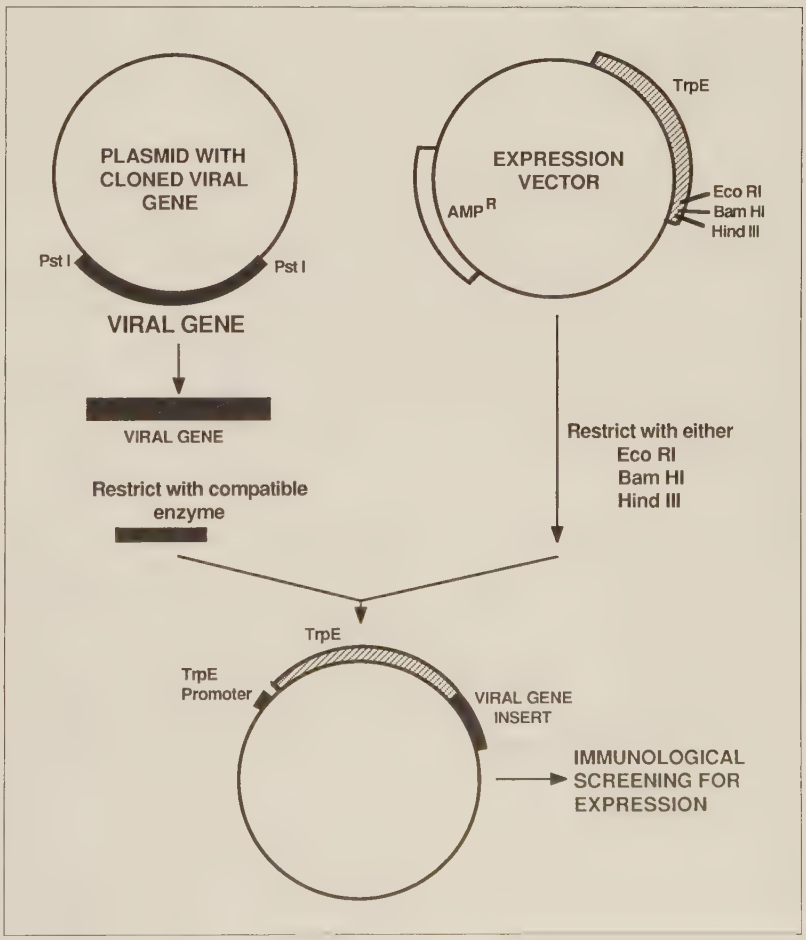

Figure 2
Construction of the expression vectors for *trp*E-viral gene fusions. The cDNA cloned insert of the IHNV glycoprotein gene or the A segment of the IPNV genome was restricted with a compatible nuclease to permit the insertion of a portion of the viral gene into the expression vector in the proper reading frame. The resulting plasmids were used for expression of a *trp*E-viral gene fusion protein in *E. coli*.

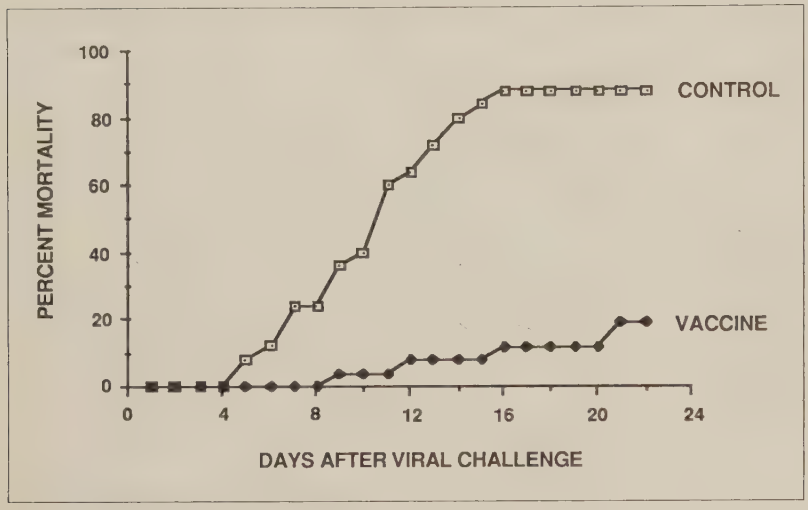

Figure 3
Immunization of rainbow trout with a subunit vaccine against IHNV. Rainbow trout fry (0.4 g) were immersed in a bacterial lysate (3 mg/mL, containing 10% expressed *trp*E-G fusion protein) as described in Materials and Methods section. The results are expressed as mean percent total mortality on the ordinate and days after the initiation of viral challenge on the abscissa. There were 25 fish in the control group and 26 fish in the vaccinated group. In this particular challenge, the fish received $7.2 \times 10^{+5}$ plaque forming units/mL.

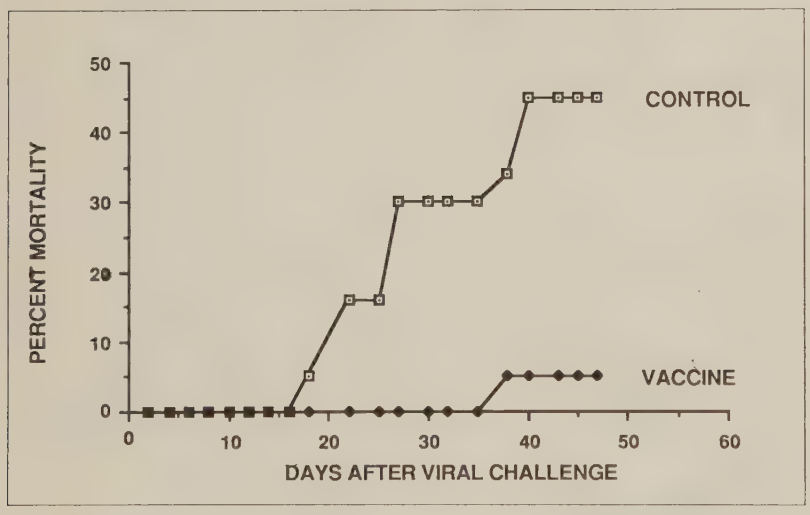

Figure 4
Immunization of rainbow trout with a subunit vaccine against IPNV. Rainbow trout fry (0.3 g) were immersed in a bacterial lysate (3 mg/mL) containing the *trp*E-VP2 fusion protein as described in Materials and Methods section. The results are expressed as percent total mortality on the ordinate and days after the initiation of viral challenge on the abscissa. There were 25 fish in both control and vaccinated groups. The fish received 10^{+6} plaque forming units/mL of IPNV-Buhl strain for viral challenge.

of IPNV-Sp were expressed by this recombinant plasmid in bacteria.

Immunization Trials with Subunit Vaccine

Viral challenges provided data on the efficacy of the bacterially expressed protein as vaccines. A significant level of protection (69%) was conferred on fish immunized with p52G versus unimmunized fish when challenged with the Round Butte isolate of IHNV (Figure 3). The glycoprotein used in constructing the fusion protein was derived from this strain. In Figure 4, the protection that was achieved by immunization with pTA1 against the Buhl isolate of IPNV, a heterologous virus strain, is shown. A decrease in virus-induced mortalities from 45% to 3% was found for the immunized group of fish.

Discussion

We have presented initial findings on the efficacy of bacterially expressed viral proteins as subunit vaccines for fish. Both the IHNV and the IPNV vaccines were effective in immunizing fish against lethal viral challenge in laboratory trials. Moreover, the vaccinations were carried out on rainbow trout fry that were 0.4 g in size. These fish were able to respond effectively to the viral vaccine. Previous studies of immunization in fish have indicated that the minimum size for successful immunization by immersion was 0.8 g for chinook salmon (Fryer et al. 1978) and 1–2.5 g for rainbow trout (Johnson et al. 1982).

The use of these vaccines with different species of fish and against a variety of different viral strains must be tested. In addition, the duration of effective immunity must be determined. However, the possibility now exists for

developing an inexpensive and effective vaccine for fish using recombinant DNA technology.

The development of any vaccine must have safety as well as efficacy as one of its primary considerations. The safety of live attenuated vaccines has been questioned for the aquaculture industry because of the nature of the environment where the vaccine would be applied. The vaccine has to be completely safe for cultured and wild salmonid fish in the watershed. Moreover, the vaccine has to be economical and a subunit vaccine produced in bacteria seems to be a viable alternative. The initial trials of the subunit vaccines reported here suggest that bacterially expressed viral proteins, even in crude lysates, can be used as effective and economical viral vaccines.

Acknowledgments

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Genetic Monitoring of Pacific Salmon Hatcheries

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ABSTRACT

In the last few decades, and in response to substantial reductions in the abundance of wild populations of Pacific salmon, an enormous amount of resources in both Asia and North America has been devoted to artificial propagation programs. Several factors increase the possibility of rapid (often detrimental) genetic change in cultured populations, but genetic considerations are often overlooked in the effort to increase short-term productivity. Here, we discuss recent studies using electrophoretic data for chinook salmon, *Oncorhynchus tshawytscha*, that address three important concerns for hatchery populations: levels of genetic variability, stability of allele frequencies, and genetic interactions (due to straying or overplanting) between hatchery and wild populations. Results indicate that although there is no evidence for a general reduction in levels of genetic variability in hatchery stocks relative to wild populations from the same geographic area, allele frequencies over a period of one generation changed much more in samples from hatchery populations in Oregon than in nearby wild populations. The genetic changes in the hatchery stocks appear to be due to a combination of two factors: genetic drift due to reduced effective population size, and (in some cases) the infusion of genes from other populations through straying or transfer of broodstock between hatcheries.

Introduction

As a consequence of increased fishing pressure, loss of spawning habitat, and blockage of migratory routes, returns of wild anadromous salmonids in the Pacific Northwest have declined substantially in this century. In part to mitigate these losses, an extensive public hatchery system has been developed during the last several decades. Throughout most of this period, management practices at the hatcheries have been dictated primarily by production demands, and relatively little consideration has been given to the genetic quality of released fish and their effects on wild fish. The availability of large amounts of data produced by protein electrophoresis over the last decade has made possible a critical evaluation of the genetic status of Pacific coast hatchery populations of salmonids. Here, we summarize results from several recent studies which are pertinent to three important concerns: 1) levels of genetic variability found in hatchery and wild populations; 2) stability of allele frequencies in hatchery and wild populations; and 3) genetic interactions (due to straying or overplanting) between hatchery and wild populations.

Materials and Methods

The electrophoretic data discussed here were collected over the last decade at the National Marine Fisheries Service laboratory in Seattle. A considerable database exists for all the North American species of Pacific salmon, *Oncorhynchus*, but here we consider only data for chinook salmon, *O. tshawytscha*; for this species, data are available for populations from California to Alaska. Whole juvenile fish or tissue samples (muscle, liver, eye, heart) from adult fish were collected in the field and stored at $-70°C$ until analyzed. Starch gel electrophoresis was performed as described by Aebersold et al. (1987). Each sample was surveyed for genetic variation at up to 100 presumptive gene loci, and genotypes inferred from the phenotypic banding patterns (see Utter et al. 1987 for discussion) were used to compute allele frequencies and a variety of standard indices of genetic variability and differentiation.

Levels of Genetic Variability

Recent policy statements (e.g., Northwest Power Planning

Council, 1987) regarding anadromous salmonids express two major concerns: that existing levels of genetic diversity be maintained, and that unique gene pools be preserved. Loss of genetic variability is a real concern for managed populations because constraints on money, space, and other resources often limit the size of the breeding population. In a closed population, approximately $1/2N_e$ of the existing genetic variation is lost each generation, with N_e being the effective number of breeders (Crow and Kimura 1970). The effective population size (N_e) is less than the actual number (N) if the sex ratio is uneven or if the variance in reproductive success among families is large—both factors that might be influenced by hatchery management procedures. Furthermore, if population size changes over time, long-term N_e is determined primarily by the effective number of breeders in the generation(s) with smaller size. Therefore, a population bottleneck (reduced effective breeding size in one or a few generations) can contribute appreciably to the long-term erosion of genetic variability.

To determine whether these effects are important in Pacific salmon, we examined two measures of genetic variability (average heterozygosity and effective number of alleles per locus) in a series of hatchery and wild populations of chinook salmon. The occurrence of consistently lower levels of genetic variability in hatchery stocks would suggest that artificial propagation has caused population bottlenecks. The heterozygosity data, however, provide no evidence of the erosion of genetic variability in cultured populations of chinook salmon in the Pacific Northwest. In each case where data are available for a comparison (Fig. 1), hatchery and wild populations from the same area have very similar levels of heterozygosity. This result differs from that reported in a number of studies of Atlantic salmon, *Salmo salar*, and rainbow, *Oncorhynchus mykiss*, cutthroat, *O. clarkii*, and brown trouts, *Salmo trutta* (review, Allendorf and Ryman 1987); some cultured populations of these species have been found to have greatly reduced levels of heterozygosity relative to the ancestral wild stocks.

Some interesting trends are apparent in the heterozygosity data for chinook salmon but these relate to geographic differences rather than to differences between hatchery and wild populations. In the Columbia River basin, coastal populations have higher heterozygosity than do lower river populations, which in turn retain more genetic variability than Snake River populations from farther upstream (Fig. 1). Populations from the Klamath and upper Fraser rivers also show reduced levels of genetic variability relative to those closer to the coast (Georgia Strait, Puget Sound). Presumably, these differences reflect the essentially independent evolutionary histories of the different areas and, perhaps, the smaller population size or increased frequency of population bottlenecks in the upriver populations (Winans 1989).

One drawback to the above analysis is that average heterozygosity is not very sensitive to the presence or absence of uncommon alleles. Although they contribute little to the measurement of heterozygosity, such alleles are potentially very important to a population because they allow a greater degree of plasticity in response to changes in the environment. The presence of numerous alleles (even those at low frequency) in a population ensures that each generation, many genotypic combinations are produced upon which natural selection might act. Because alleles at low frequency are easily lost if the effective breeding size is small, the average number of alleles per locus is a more sensitive indicator than average heterozygosity of undesirable changes in the genetic makeup of a population. According to Utter et al. (1989), the average number of alleles per locus for seven hatchery and six wild populations from Oregon were similar (1.74 and 1.68, respectively). This lends additional support to our conclusion that the wholesale reduction of genetic variability reported in some hatchery populations of *Salmo* (e.g., Stahl 1983) has apparently not occurred in chinook salmon hatcheries in the Pacific Northwest.

This result is encouraging, but by no means constitutes a clean bill of health for hatchery populations. If the genetic makeup of the source populations is to be perpetuated as accurately as possible, it is important not only to conserve overall levels of genetic variability, but also to avoid large changes in frequency of the alleles present. For example, consider a locus with two alleles (A and a), sampled in a population at two times, with the following frequencies observed—time 1: A = 0.8, a = 0.2; time 2: A = 0.2, a = 0.8. Hardy-Weinberg expected heterozygosity (2Aa = 0.32) remains unchanged, but allele frequencies have shifted drastically. Clearly, it is also important to monitor allele frequencies over time in artificially propagated populations.

Temporal Stability of Allele Frequencies

To evaluate the temporal stability of allele frequencies, we examined electrophoretic data for 21 coastal chinook salmon populations from Oregon and California that were sampled in each of two years (Waples and Teel 1990). For each population, allele frequencies in the two samples were compared at an average of 10 polymorphic loci. For each locus, a contingency chi-square test was used to test the hypothesis that the population frequencies were unchanged. Results of these tests are very revealing (Table 1). For the three California hatchery and the nine Oregon wild populations, the number of single locus tests showing a significant change in allele frequency (1/16 = 6% to 7/88 = 8%) was close to the value (5%) expected to arise from sampling error, while the figure for the nine

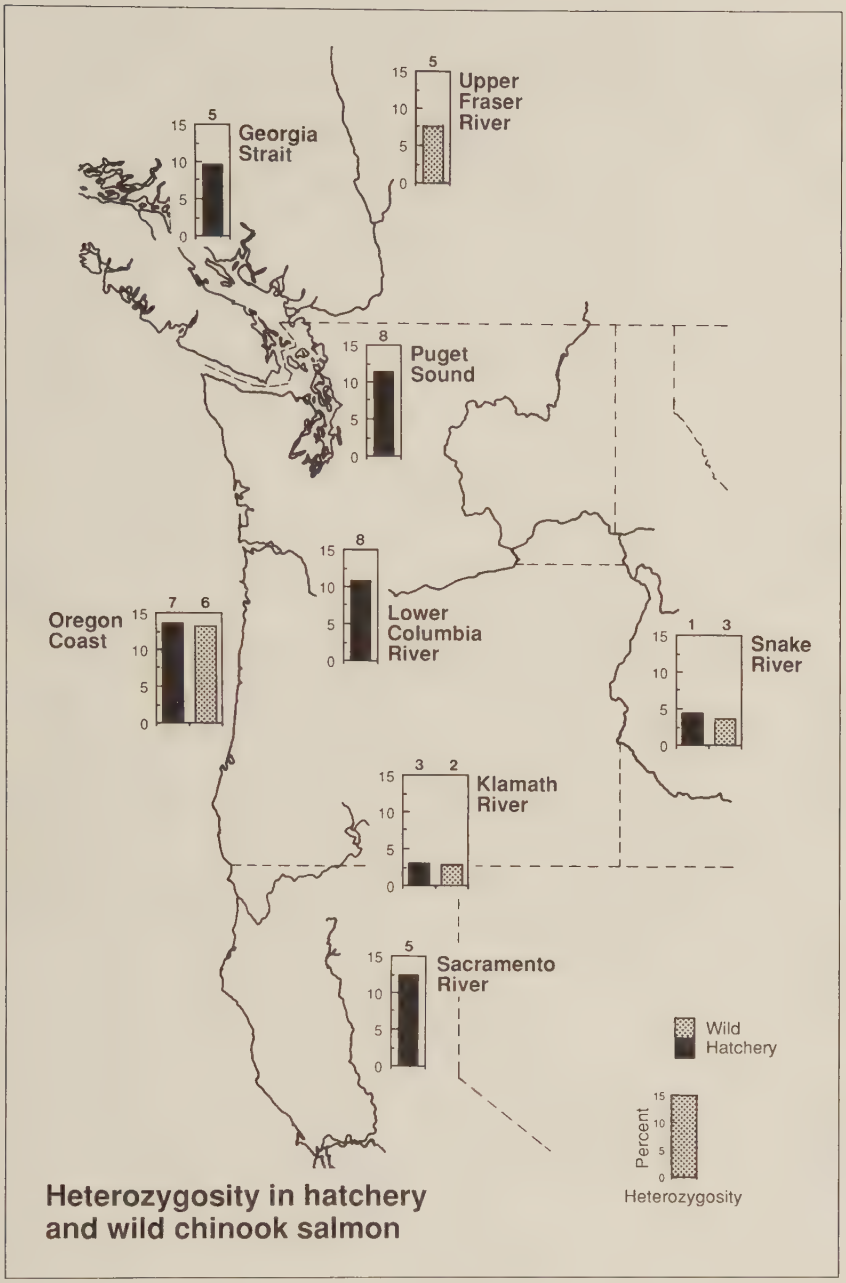

Figure 1
Comparison of average heterozygosity values for hatchery and wild stocks of chinook salmon by geographic area.

Oregon hatchery populations was much higher (29/81 = 36% of all tests showing significant allele frequency change). In addition, combined chi-square tests over all loci indicate very significant ($P<0.01$) or highly significant ($P<0.001$) changes in allele frequency between 1981 and 1985 samples in eight of the nine Oregon hatcheries (Table 1).

Possible causes of short-term allele frequency change include natural selection, genetic drift, and migration. In the present example, selection appears to be an unlikely cause, given Waples and Teel's (1990) demonstration that it is necessary to invoke unrealistically large selection coefficients to explain such large frequency shifts in a single generation. Waples and Teel also showed that the observed differences can be accounted for by genetic drift only if the effective number of breeders in the Oregon hatcheries averaged about 50 or less. Examination of brood stock data indicates that effective population size may indeed have been quite low in at least some of the hatcheries (Waples and Teel 1990).

Another possibility is that some of the genetic changes resulted from the infusion of new genes during the transfer of fish between hatcheries, or from natural straying into the hatcheries. Evidence to support this hypothesis comes

Table 1

Twenty-one chinook salmon populations sampled in each of two years. Number of significant ($\alpha = 0.05$) single locus chi square tests comparing allele frequencies in two years are shown, and significance levels are given for combined chi-square test over all loci and a test for gametic disequilibria (n.s. = not significant).

			Between-year cmparisons[c]		Gametic disequilibria[d]	
	Sample size		Single locus			
Population[a]	1981	1985[b]	(no. sig./total)	All loci	1981	1985[b]
Oregon wild populations						
Alsea	94	50	0/11	n.s.	n.s.	n.s.
Chetco	100	93	1/7	n.s.	n.s.	n.s.
Coquille	115	50	1/12	n.s.	n.s.	n.s.
Nehalem	141	50	1/9	n.s.	n.s.	n.s.
Nestuca	60	50	1/9	n.s.	n.s.	n.s.
Siletz	92	50	0/11	n.s.	n.s.	n.s.
Sixes	100	50	1/8	0.01	n.s.	n.s.
Siuslaw	82	34	1/11	n.s.	n.s.	n.s.
Tillamook	88	50	1/10	n.s.	n.s.	n.s.
No. sig./total			7/87	1/9	0/9	0/9
Oregon hatchery populations						
Cedar Creek	99	100	4/9	0.001	0.01	0.001
Cole R. (S)	113	50	1/9	n.s.	n.s.	n.s.
Cole R.	50	100	5/13	0.01	n.s.	n.s.
Elk R.	100	100	2/9	0.001	n.s.	n.s.
Fall Creek	100	100	2/7	0.01	0.001	n.s.
Rock Creek (S)	100	100	4/9	0.001	0.05	0.001
Salmon	99	100	5/8	0.001	n.s.	0.001
Trask (S)	100	100	3/10	0.001	n.s.	0.05
Trask	100	100	3/7	0.01	0.01	0.001
No. sig./total			29/81	8/9	4/9	5/9
California hatchery populations						
Iron Gate	99	50	1/8	n.s.	n.s.	n.s.
Trinity (S)	50	100	0/5	n.s.	—	0.05
Trinity	100	50	0/3	n.s.	n.s.	n.s.
No. sig./total			1/16	0/3	0/2	1/3

[a] Spring run denoted by (S); all others are fall run stocks.
[b] Samples taken in 1983 for Oregon wild populations, 1984 for California populations.
[c] Data from Waples and Teel (in press).
[d] Data from Waples and Smouse (1990).

from gametic disequilibrium analysis, a powerful means of detecting samples which are actually a mixture of distinct gene pools. Gametic disequilibrium (the nonrandom association of alleles at different gene loci) occurs as the result of a mixture of gene pools that differ in allele frequency at two or more loci (Nei and Li 1973).

Genetic Interactions Between Hatchery and Wild Populations

Admixtures (mixtures of fish from more than a single gene pool) involving hatchery populations are a concern for two reasons. First, the transfer of eggs, fry, and brood stock among hatcheries is a common occurrence that complicates the problem of identifying the genetic makeup of hatchery populations. Second, strays of hatchery or transplanted fish may have an adverse effect on wild populations adapted to local conditions. The genetic consequences of such admixtures are difficult to evaluate by traditional methods (physical tags, behavioral observations) because the presence of exotic fish in a population does not ensure that they will interbreed with the residents and produce viable offspring. If the potential source populations can be identified and adequate genetic markers are available, estimates of the mixture fractions are possible (Campton 1987). However, in many cases the populations possibly contributing

to a mixture are unknown or cannot be characterized genetically. For such a "blind" mixture, gametic disequilibrium analysis is a potentially powerful tool for evaluating the null hypothesis that the sample could have come from a single gene pool.

To evaluate the possibility of genetic admixture in the above example, we used a multilocus analysis of gametic disequilibrium (Waples and Smouse 1990) that considers data for all pairs of loci simultaneously. No unusual levels of multilocus gametic disequilibrium were found in the California hatchery or Oregon wild populations (1 of 23 samples with significant disequilibria at $\alpha = 0.05$ level; Waples and Smouse 1990), but the situation was quite different in the samples from the Oregon hatcheries (9 of 18 tests significant; Table 1). These findings are consistent with the hypothesis that, in addition to genetic drift, a mixture of gene pools contributed to the changes in allele frequency observed in some of the Oregon hatchery populations.

Gametic disequilibrium analysis has considerable potential for assessing the extent of genetic interactions among hatcheries and between hatchery and wild populations. It may help in the identification of wild gene pools that have been relatively unaffected by genes from hatchery populations and therefore merit conservation efforts. In other cases, where the objective is to supplement and enhance wild production, gametic disequilibrium analysis provides a means of monitoring the effectiveness of transplants from the hatcheries. The possibilities for both types of genetic interactions, as well as the necessity for monitoring them, are likely to increase in the near future. Large scale supplementation of wild populations and expansion of hatchery production are planned to achieve the goal of the current Columbia River Basin Fish and Wildlife Program (Northwest Power Planning Council 1987)—doubling the run size of anadromous salmonids in the Columbia River basin.

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Successful Gene Transfer in Fish

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ABSTRACT

The *neo* gene, which confers resistance to the neomycin analog drug G418, has been successfully transferred into newly-fertilized dechorionated goldfish eggs by microinjection. Multiple copies of the gene were incorporated into the high molecular weight fraction of fish DNA (i.e., the genomic DNA). RNA dot blots indicate specific *neo* mRNA synthesis. Gene transfer efforts using mammalian-derived growth hormone genes, a marker gene for β-galactosidase, and a variety of promoters are discussed. Current work which also includes the isolation of piscine promoters and genes for peptide hormones is mentioned.

Introduction

Novel genes were first introduced into mice in 1979 by Gordon et al. (1980). The technology did not receive wide attention until Palmiter et al. (1982) transferred a rat growth hormone gene linked to a mouse metallothionein promoter, creating a line of mice that grew significantly faster and ultimately larger than control mice. This series of experiments captured the imagination of a wide variety of researchers seeking to improve economic traits in domestic and semidomestic animals. While gene transfer experimentation is currently progressing in most species of economic importance, including mammalian farm animals and poultry, no group of organisms shows more promise for the dramatic interaction of transferred genes than fish. For over three decades it has been known that fish are quite responsive to injections of crude and purified growth hormone (Pickford and Thompson 1948; Adelman 1977). Successful transfer and expression of growth-hormone genes in fish are thus expected to produce a similar, dramatic response.

Since 1984, multiple groups worldwide have been pursuing the goal of producing transgenic fish. Laboratories in Japan, England, France, and the People's Republic of China have published results of these attempts in medaka, *Oryzias* sp. (Ozato et al. 1986), rainbow trout (*Oncorhynchus mykiss*) (Maclean and Talwar 1984; Chourrout et al. 1986), goldfish (*Carassius auratus*) (Zhu et al. 1985), and loach (*Misgurnus anguillicaudatus*) (Zhu et al. 1986). Within the United States, four primary groups are known to be using microinjection of various plasmid constructs to produce transgenic fish. These laboratories are, in addition to our group at the University of Minnesota, those of Drs. Powers and Chen at Johns Hopkins, Dunham at Auburn, and Ellinger and Kohler at Southern Illinois. All four U.S. groups are pursuing similar goals: production of transgenic fish by transfer of growth-hormone gene constructs using microinjection as the primary transfer technique. Our group reports here the successful transfer of a marker gene, *neo*, into goldfish via microinjection, as a step toward the goal of transfer of economically important genes.

Materials and Methods

Egg Preparations

Spontaneous ovulation of goldfish was accomplished by the methods of Stacy et al. (1979). Sexually mature fish were kept under a long photoperiod (16 h light, 8 h dark). On day one, breeding fish were transferred from stock aquaria to standing-water breeding aquaria at 13 ± 1°C. Aquaria were supplied with floating artificial plants, and the water temperature was increased to 21 ± 1°C overnight and kept at that temperature through spawning. Spontaneous ovulation usually occurred during the last half of the dark phase on day 3. If no ovulation occurred on day 3, the fish were injected intraperitoneally with 3 mg/kg body weight of carp pituitary gland extract. About 10 hours later, the fish were

artificially spawned and eggs and sperm were collected separately.

Eggs were fertilized by the milt after they were mixed with well water in an open petri dish in batches of approximately 100 eggs. Ten minutes after fertilization, the eggs were dechorionated by a six minute incubation in 0.2% trypsin (Zhu et al. 1985). Dechorionation was stopped by treatment with 5% fetal bovine serum in Holtfreter's solution (Grand et al. 1941). Dechorionated eggs were washed several times in Holtfreter's solution and transferred to Holtfreter's solution on a charcoal-agar petri dish, which provided a dark background for microinjection.

Microinjection

Plasmids were prepared by amplification, lysis in SDS (sodium dodecylsulfate), and $CsCl_2$ centrifugation (Maniatis et al. 1982). Plasmids were linearized with restriction endonuclease *Kpn*I, extracted with phenol/chloroform, ethanol precipitated, and redissolved in 88 mM NaCl, 10 mM Tris·Hcl pH 7.6 to a final concentration of 25 ng/μL. Borosilicate glass needles with an inner tip diameter of approximately 2 μm were filled with plasmid solution. Microinjection was performed with a Brinkman MM33 micromanipulator with the injection volume controlled by the timing of the insertion/withdrawal interval and constant fluid flow. It is estimated that 2 nanoliters of solution containing 1×10^6 copies of *neo* gene are delivered. DNA was released into the center of the germinal disc prior to the first cleavage. Microinjected eggs were allowed to develop in Holtfreter's solution until the blastula stage, and returned to well water after the blastula stage. Mock injections were performed as controls, using only the buffer component of the injection solution.

Plasmid Construction

The primary construct used, pRSVneo, contains the *neo* gene, whose product, aminoglycoside 3′-phosphotransferase, confers resistance to the neomycin analog G418, and a Rous sarcoma virus (RSV) promoter. A *Bam*HI-*Hind*III restriction fragment containing the *neo* gene and mart of SV40 intron was isolated from pSV2neo (American Type Culture Collection) and ligated into the polylinker of pUC119. A 340-bp fragment containing the RSV promoter/enhancer region was ligated to the regenerated *Hind*III site. This construct is termed pRSVneo in this paper. Plasmid construction was by standard methods (Maniatis et al. 1982).

DNA Analysis

Genomic DNA was isolated from posterior halves of 1 to 2 month-old (approx. 8 g) fish stored at −90°C. Fish were individually homogenized in three volumes of 0.2 M Tris·HCl pH 8.0, 0.1 M EDTA, 0.5% SDS buffer with 100 μg/mL proteinase K on ice using a Dounce homogenizer. High molecular weight DNA was extracted as per Maniatis et al. (1982).

DNA dot blot analysis was used to detect the *neo* gene. Approximately 5 μg of genomic DNA were denatured in 0.4 M NaOH, and following addition of an equal volume of 2 M ammonium acetate, were spotted onto nitrocellulose using a Hybri-dot system. The filter was baked *in vacuo* for 2 hours at 80°C. The filter was prehybridized in 5 × SCC (1 × SSC = 0.15 M NaCl, 15 mM Na citrate), 5 × Denhardt's solution (1 × Denhardt's = 0.02% Ficoll, 0.02% bovine serum albumin, 0.02% polyvinylpyrrolidone), 50 mM sodium pyrophosphate pH 6.5, 0.1% SDS, 0.1 mg/mL denatured calf thymus DNA, and 0.1 mg/mL yeast tRNA at 65°C for at least 4 hours. The filter was hybridized with 1×10^6 cpm of ^{32}P labeled probe in the same solution used for prehybridization for 20 hours at 65°C. The filter was washed three times in 2 × SSC, 0.1% SDS at room temperature for 10 minutes, and three times in 0.1 × SSC, 0.1% SDS at 60°C for 30 minutes, dried, and exposed to Kodak XAR5 x-ray film at −80°C (Maniatus et al. 1982).

Southern blot analysis was performed by complete digestion of 10 μg of genomic DNA with *Kpn*I, *Bam*HI, *Sst*I, or *Mlu*I followed by electrophoresis on a 0.8% agarose gel. Transfer to nitrocellulose was made following the method of Southern (1975). The Southern blot was probed using the same hybridization conditions as the dot blot analysis.

RNA Analysis

Anterior halves of frozen (−90°C) *neo* DNA positive fish plus several control and injected fish were homogenized by Polytron in a solution containing 4 M guanidinium isothiocyanate, 0.1 M β-mercaptoethanol, 0.5% sarkosyl, 5 mM sodium citrate, pH 7.0. Total RNA was isolated as per Maniatis (1982). RNA dot blot analysis was used to test for *neo* gene expression. Total RNA was dissolved in 6 × SSC, and 7.4% (v/v) formaldehyde, and heat denatured at 65°C for 15 minutes. Serial amounts of RNA (0.25, 0.5, 1, and 2 μg) were directly dotted onto nitrocellulose equilibrated with 6 × SSC. The RNA was fixed to the filter by baking under vacuum at 80°C for 2 hours. The prehybridization was carried out for more than 4 hours at 60°C in 50% deionized formamide, 5 × SSC, 5 × Denhardt's solution, 50 mM sodium phosphate, pH 6.5, 0.1% SDS, 100 μg/mL denatured calf thymus DNA, and 100 μg/mL yeast tRNA. The filter was then hybridized with 2×10^6 cpm/mL of ^{32}P-labeled probe in the same buffer for 20 hours at 60°C. After hybridization, the filter was washed three times with 2 × SSC, 0.1% SDS at room temperature for 10 minutes, three times with 0.2 × SSC, 0.2% SDS at 68°C for 30 minutes, and exposed to Kodak XAR5 x-ray film at −80°C.

Figure 1

DNA dot blot analysis of goldfish microinjected with pRSVneo. Total genomic DNA probed with 330 bp *Hind*III-*Bam*HI restriction fragment of the promoter region of the *neo* gene. Rows 7 and 8 present dilutions of pRSVneo with (row 8) or without (row 7) added genomic DNA. Dilution series is A–B, 0.5 copies; C–D, 1 copy; E–F, 5 copies; G.H, 25 copies; and K–L, 100 copies per genome. Test fish DNA was spotted twice on filter producing series 1-5, A–F and an identical replicate 1–5, G–L. Dots row 1A–1F (replicate 1G–1L) are of six noninjected control goldfish. Dots row 2A–2F and 3A–3E (replicate 2G–2L and 3G–3K) are of pRSVneo-injected goldfish DNA. Dots 3B and 3D indicate presence of approximately five copies of *neo* gene. Dots 3F, 4A-4F, and 5A-5C (replicate 3L, 4G–4L, and 5G–5I) are of pRSVneo-injected fish subjected to G-418 drug selection (4 mg/mL), arranged in increasing survival time from 20 hours (3F) to greater than 8 days (5C). Dots 5D–5F are test dots for detection of possible neo-hybridizable sequences in pink salmon DNA.

Probe Labeling

Plasmid restriction fragments were separated by 1% agarose gel electrophoresis in TAE buffer (0.04 M Tris-acetate, 2mM EDTA pH 8.0). Excised bands were phenol:chloroform extracted, and the DNA was precipitated by ethanol and sodium acetate and redissolved in TE (10 mM Tris·HCl pH 7.6, 1 mM EDTA). Fragments were labeled by the procedure of Feinberg and Volgelstein (1983) to $0.5–2 \times 10^9$ cpm/μg. DNA probes were used without purification from unincorporated nucleotides.

The 2.3-kb fragment including the *neo* gene was also subcloned into pTZ18R under the control of the T7 promoter to produce a transcript complementary to *neo* mRNA. One μg of linear DNA was transcribed using the method of Schenborn and Mierendorf (1985) to produce probes used in RNA dot blot analysis.

G-418 Selection

For the drug selection screenings, solutions of G-418 in static water aquaria were aerated to achieve initial saturation levels of dissolved oxygen. Dissolved oxygen was maintained above 60% saturation by periodic aeration. The temperature was maintained at 20 ± 1°C. The pRSVneo-injected or non-injected control goldfish at a population density of 12 g/L were exposed to 4 mg/mL solutions of G-418. Mortality of fish was monitored every four hours for the first 24 hours and every 12 hours afterward. Dead individuals were removed upon observation and stored in liquid nitrogen. Drug selection was continued until 50% of the control fish in 4 mg/mL G-418 had died. Surviving fish were transferred into fresh well water upon termination of the test.

Results

The survival rate for microinjected fish has ranged from 10% in the early experiments to nearly 50% at the current time. The results reported here are from early injection studies, where, despite the high mortality rates, we were still able to effect the transfer and apparent expression of the transferred gene.

Figure 1 shows the results of DNA dot blot analysis of mock- and pRSVneo-injected goldfish. None of the mock-

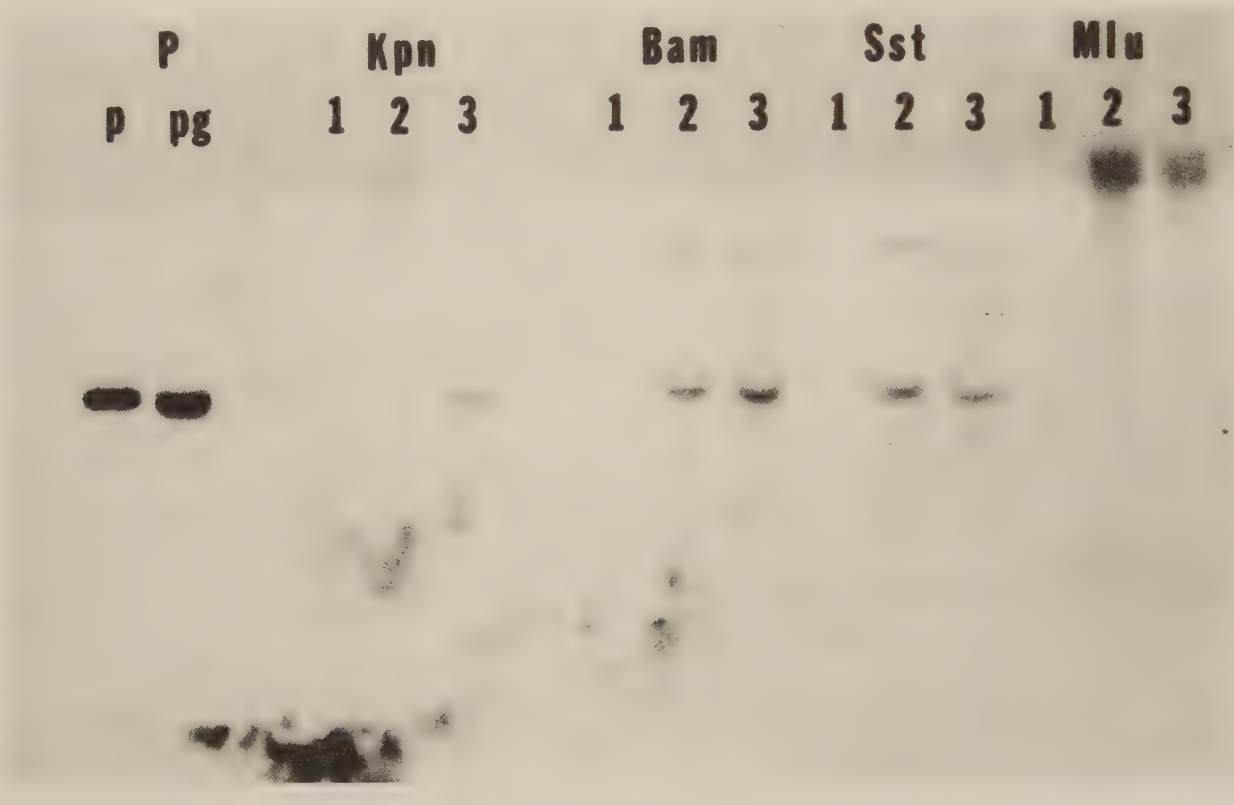

Figure 2

Southern analysis of genomic DNA from three goldfish microinjected with pRSVneo. Fish #1 was negative by DNA dot blot analysis, while fish #2 and #3 represent fish from dots 3B and 3D of Figure 1. Lanes p and pg represent linearized pRSVneo and pRSVneo plus genomic DNA, respectively.

injected fish showed positive hybridizaiton to the *neo* probe, while two of the pRSVneo-injected fish showed approximately five copies of the gene per genome. Figure 1 also shows a series of DNA dot blots for pRSVneo-injected fish that were subjected to drug selection with G-418. The fish were grouped by order of the time of death during drug selection. One fish, which survived the full course of selection, but died on day 8 of fungal infection, showed an apparent hybridization signal of about 1 copy per genome. Pink salmon DNA was included as control DNA. Note that only the pink salmon DNA dot blots of Figure 1 (Dots 5D–5F), show high background hybridization to the probe in both control and injected fish. Unlike goldfish, pink salmon apparently contain genomic sequences that are closely related to *neo*.

Figure 2 shows results of a Southern blot analysis of genomic DNA cleaved with four different restriction enzymes and probed with the 330-bp *neo* probe. The *neo* probe detected both linearized plasmid and a higher molecular weight band in the *Bam*HI and *Sst*I digests. This may indicate a concatenated integration of the estimated five copies of the gene, but conclusive evidence of integration will require breeding studies.

Expression of the transferred *neo* gene which was suggested in the resistance of a fish containing *neo* sequences (Fig. 1), was confirmed in the RNA dot blot of Figure 3. Here, one out of six fish tested showed strong expression of *neo* RNA when probed with a complementary *neo* RNA probe. In order to rule out the possibility of DNA contamination of the RNA used in the RNA dot blot, we treated the RNA from the positive fish of Figure 3 with DNase and RNase in a separate experiment (Fig. 4). RNase destroyed the hybridization to the *neo* RNA probe, while DNase had little effect. This indicates little if any DNA contamination of the RNA.

Conclusion and Discussion

The results reported here showed successful transfer of a marker gene *neo* that encodes resistance to the drug G-418. Stable integration of the transferred gene was suggested but could not be conclusively proven at this time. Expression of the marker gene was confirmed through detection of *neo* mRNA in the transgenic fish. Functional resistance conferred by pRSVneo was suggested by the survival through drug selection of a putative transgenic fish containing an extimated single copy of the *neo* gene.

These results indicate that microinjection is a viable method for transfer of selectable genes into fish. Micro-

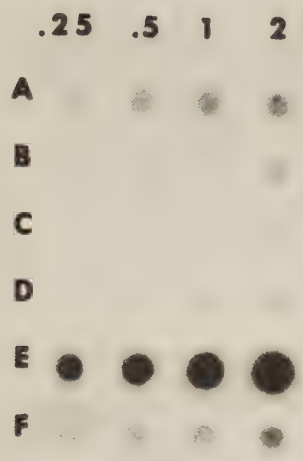

Figure 3
RNA dot blot analysis of total RNA from six pRSVneo-injected fish, spotted at 0.25, 0.5, 1 and 2 μg RNA per dot for each fish.

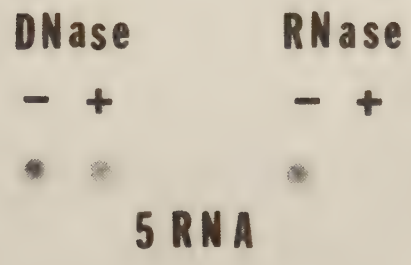

Figure 4
Total RNA from fish E of Figure 3 probed with RNA complementary to *neo* RNA transcript. RNA was spotted before (–) and after (+) treatment with RNase-free DNase or DNase-free RNase.

injection is, however, currently a labor- and patience-intensive technique, and promises to remain so for the near future. Microinjection works well upon dechorionated eggs where needle placement can be monitored, but is less effective in blind injections. Therefore, we are keenly interested in development of a selectable marker for use in either blind injection or in mass transfer techniques. Our group has been exploring the use of mass transfer techniques to overcome the tedium of injection of the substantial numbers of eggs necessary to generate the optimal transgenic fish. While mass transfer efficiencies may be orders of magnitude less than those achieved by microinjection, the ability to manipulate tens of thousands of eggs at a time should compensate for the loss in transfer efficiency. The key element necessary to make mass transfer a reality is the development of a selection system to differentiate transgenic from nontransgenic fish. The selectable marker *neo* meets this criterion, and should prove effective in the selectionof transgenic fish when used in co-transfer schemes with other genes.

A variety of mass transfer techniques are under investigation by our group as well as by other laboratories in the United States. These range from techniques based on the $CaPO_4$ procedures used in gene transfer in tissue culture, to binding of plasmid DNA to sperm and effecting transfer at the time of fertilization, to electroporation. Studies proceeding along these lines should soon yield general mass transfer protocols that will be successful in a broad spectrum of fish species.

The establishment of the *neo* gene as a selectable marker is a step in the generation of transgenic fish with enhanced growth hormone expression. We are currently injecting bovine growth hormone constructs into fish eggs, as well as injecting a second marker gene construct using β-galactosidase. While bovine growth hormone gene constructs may produce growth enhancement in transgenic fish, our intent is to effect growth enhancement by transfer of species-specific growth hormone genes. To this end, we are isolating piscine growth hormone genes and fish gene promoters so that the ultimate transgenic, growth-enhanced fish will contain no DNA sequence from outside its own species. We feel that this will simplify regulatory approval of the transgenic fish for human consumption, and enable the transferred genes to interact with the natural regulatory mechanisms in the fish in a more normal fashion.

Acknowledgments

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Clonal Ginbuna Crucian Carp as a Model for the Study of Fish Immunology and Genetics

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ABSTRACT

The lack of suitable inbred lines of fish for immunological study in which lymphocytes and blood samples could be collected have led to the use of clonal ginbuna crucian carp, *Carassius gibelio langsdorfii*. Distributed widely in Japan they include both bisexual diploid and tetraploid types and a unisexual (all female) triploid type which reproduces gynogenetically by omitting Meiosis I. The kinetics of immunity transfer by immune leukocytes was first examined using isogeneic crucian carp. Splenic cells were most effective in transferring immune reactivity, followed by pronephric, then mesonephric cells. However, antibody titres were very low or absent when the recipients received transferred thymic cells. Antibody productivity was most successfully conferred when cells were taken from 7-day postimmunized donors. The level of antibody titre in recipients reached its peak on day 7 for mesonephric cells and on day 14 for pronephric and splenic cells. Transferability of immune leukocytes was also compared in isogeneic, allogeneic and xenogeneic crucian carp to examine their major histocompatibility complex (MHC) regulation in adoptive immunity. Adoptive transfer by pronephric cells was successful in isogeneic and weak histocompatibility (H) gene-disparate transfer systems, while antibody productivity was not transferable in xenogeneic and strong H gene-disparate transfer systems. In allogeneic fish, however, antibody productivity was transferable by the transfer of cells in some recipients that rejected the allograft in an acute fashion.

Introduction

Most of the information on the cellular immune mechanism in mammals has been obtained by in vivo and in vitro experiments using histocompatible animals. In fish, however, suitable inbred lines for immunological study are not available. Although inbred strains of small teleosts have been established by sibmating (e.g., platyfish and swordtails, *Xiphophorus* spp., (Kallman 1964); guppy, *Lebistes reticulatus*, (Schroder and Holzberg 1972); and medaka, *Oryzias latipes*, (Taguchi 1980), they are too small to collect lymphocytes and blood samples. Furthermore, genetically identical animals are not currently available for practical use, even though chromosomal set manipulation techniques have seen great improvement in recent years and homozygous clonal fish have been obtained by the suppression of meitosis I and successive prevention of Meiosis II (e.g., zebra fish, *Brachydanio rerio*, (Streisinger et al. 1981), medaka (Naruse et al. 1985) and rainbow trout, *Oncorhynchus mykiss*, (Onozato 1990).

Fortunately, clonal crucian carp—naturally occurring gynogenetic fish—are distributed widely in Japan. In the present review, we describe our recent studies on the fish immune system using clonal crucian carp and discuss the excellent potential of this fish as a model for studying fish immunology and genetics.

Clonal State of Triploid Ginbuna

Naturally occurring gynogenetic populations are included in two cyprinid species: silver crucian carp, *Carassius auratus gibelio* (Cherfas 1966), and ginbuna, *C. gibelio langsdorfii* (formally *C. auratus langsdorfii*, Kobayashi 1971). Ginbuna have the widest distribution in Japan, and include both bisexual diploid (2n = 100) and tetraploid (4n = 206) types, and unisexual (all female) triploid (3n = 156) types (Kobayashi et al. 1970, 1977). In unisexual ginbuna, which reproduce gynogenetically, the first polar body formation is skipped as the result of lacking Meiosis I (Kobayashi 1976). Therefore, the progenies of these fish belong to a clone having the same genotype as the mother, as evidenced by scale grafting and electrophoreses (Onozato 1981; Murayama et al. 1984). Onozato (1981) has shown that the electro-

phoretic pattern of isozymes LDH, MDH, α-GPD and the muscle protein of all progenies coincide with that of their mother. Of most interest is the fact that ginbuna was found to be a heterozygous clone from isozyme analyses (Onozato 1983).

Natural populations of triploid ginbuna consist of several clones which differ in strong or weak histocompatibility genes (H-genes). Allografted scales reciprocally exchanged among strong H gene-disparate members were rejected within one week in an acute fashion, whereas allografts among weak H gene-disparate members remained intact until 4 to 5 weeks before rejection (Nakanishi and Onozato 1988).

Table 1

Antibody response to erythrocyte antigens in crucian carp. Fish were given three injections at 2-day intervals with 5 μL/g body weight of 20% erythrocyte intraperitoneally.

Antigens	Individual HA[a] titres ($-\log_2$)	Mean ± SD
Bovine	5, 5, 4, 1, 0	3.0 ± 2.1
Horse	12, 10.5, 10, 9.5, 9.5	10.3 ± 0.9
Sheep	7, 7, 7, 7, 6.5	6.9 ± 0.2
Guinea-pig	10, 9.5, 8, 7, 5	7.9 ± 1.8
Chicken	10, 9.5, 9, 9, 8	9.1 ± 0.7

[a] Hemagglutination.

Ginbuna Immunology and Genetics

Variability in Immune Reactivity

It is a well-known fact that immune responses are genetically controlled. Our greatest concern was to determine to what extent the variation exists in response to erythrocyte antigens in genetically identical animals. If the variation is not great, these animals could be used in various studies such as the examination of vaccines and drugs. As well as using fewer animals, we could also expect the results to be more reproducible compared to those obtained using outbred animals.

We examined several erythrocyte antigens for monitoring immune reactivity. Erythrocytes in Alsever's solution were purchased from Teikoku Zoki Ltd (Tokyo), washed three times with phosphate-buffered saline (PBS, pH 7.2) and adjusted to appropriate concentration. All animals were given an intraperitoneal injection (5 μL/g body weight) every 3 days for 6 days. Immunized fish were bled from the caudal blood vessels with an heparinized syringe, and the plasma samples were analyzed for hemagglutination titres by a microtiter method. Fish weighing 20–30 g were maintained in tanks with running water at 25 ± 1°C and fed pellet. Anesthetization was performed with ethyl p-amino benzoate (100 mg/L) prior to manipulation. Horse red blood cells (HRBC) were found to be the best antigen for inducing high titres of antibody in crucian carp (Table 1). We also examined the effect of concentration on the diversity in immune responses using outbred nigorobuna crucian carp, *Carassius carassius grandoculis*. At a high concentration of 20%, most of fish elicited similar titres. At lower concentrations, however, greater variation was ob-

Table 2

Effect of dosage HRBC (horse red blood cells) antigen on the antibody response of outbred nigorobuna crucian carp at 25°C.

Concentration of antigen	Weeks[a]	Individual HA[b] titres ($-\log_2$)	Mean	C.V.[c]
20%	2	11, 10, 10, 8.5, 7	9.3	0.15
	3	11, 10.5, 10, 8, 8	9.5	0.13
5%	2	7.5, 7, 6, 6, 0	5.3	0.51
	3	9, 7, 7, 7, 2.5	6.5	0.33
1%	2	9, 7, 5, 5, 0	5.2	0.58
	3	9.5, 7, 7, 6.5, 0	6.0	0.53
0.1%	2	7, 5.5, 4, 4, 3	4.7	0.30
	3	8, 7.5, 6.5, 6.5, 5	6.7	0.15
0.01%	2	1, 1, 0, 0, 0	0.4	1.22
	3	4.5, 3, 2.5, 2, 1	2.6	0.45
0.001%	2	0, 0, 0, 0, 0	0.0	0.00
	3	6, 4, 3, 0, 0	2.6	0.90

[a] Interval between the first injection and the bleeding.
[b] Hemagglutination.
[c] Coefficient of variance.

Table 3
Comparison of variability in antibody responses to HRBC between allogeneic and isogeneic crucian carp.

		No. of animals	Individual HA[a] titres ($-\log_2$)[b]	Mean	C.V.[c]
20%	Allogeneic	15	13(1), 12(1), 11(2), 10.5(1), 10(4) 9.5(3), 9(1), 8.5(1), 7(1)	10.0	0.14
	Isogeneic	15	13(3), 12.5(1), 12(3), 11.5(2) 11(3), 10.5(1), 10(1), 9(1)	11.5	0.10
1%	Allogeneic	15	9(1), 8(1), 7.5(1), 7(5), 6(3) 5.5(1), 4.5(2), 3.5(1)	6.4	0.22
	Isogeneic	15	8(1), 7.5(1), 7(5), 6(6), 5(1), 4(1)	6.4	0.15
0.01%	Allogeneic	10	7(2), 5(2), 4(1), 3.5(2), 3(1), 4(2)	4.2	0.41
	Isogeneic	10	10(1), 8(1), 7(2), 5(1), 4.5(1) 4(3), 3(1)	5.7	0.38

[a] Hemmaglutination.
[b] Number of fish is shown in parenthesis.
[c] Coefficient of variance.

served; some fish showed high titres, and others did not respond at all (Table 2). There is no apparent difference between allogeneic and isogeneic crucian carp even at lower concentrations (Table 3). That is, genetically identical ginbuna showed similar considerable variation to that of the outbred nigorobuna in immune responsiveness.

Similar results have been obtained from the study of genetic influence on the diversity in growth rate using clonal crucian carp (Nakanishi and Onozato 1987). In this study a great variation in size and weight was found, even in clonal crucian carp, indicating the occurrence of a superior group when the fry were reared in small tanks at high densities with food of different partical size.

These results suggest that immune reactivity or growth is not only dependent on genetic state but is greatly influenced by other factors which may have been formed in correlation with environmental circumstances. That is, individuals have met with different microenvironments throughout the life span, including egg state in the ovary, and the differences in physiological conditions must have occurred among individuals later on.

Kinetics of Transfer of Immunity by Immune Leukocytes

Adoptive transfer of immunity can be successful only when recipients show no alloimmune response. However, the histocompatibility system of fish has been found to be well developed (Kallman 1970; Borysenko 1976), with rejection often occurring in an acute fashion even among siblings. Therefore, adoptive transfer is only possible when inbred or clonal fish are used.

Fish source and the procedures of immunization, leukocyte collection, and cell transfer have been described in Nakanishi (1987a). In brief, donors were injected intraperitoneally at 2-day intervals with 5 μL HRBC/g body weight. Donors used for day-3 transfer were given a single injection, those for day-5 transfer were given two injections and those for day-7 or after were given three injections. Recipient fish were subsequently infused with lymphoid cells at a rate of 0.1 mL of 5×10^7 cells/mL per fish. Pronephric and mesonephric cells from a fish were injected into two or three fish and thymic and splenic cells were transferred into one fish. Recipients were evaluated by measuring the serum antibody titre using standard methods of hemagglutination in microtiter plates.

In a comparison of transferability between lymphoid organs, splenic cells were most effective in transferring immune reactivity, followed by pronephric, then mesonephric cells (Fig. 1). Little or no antibody titres were elicited when the recipients received transferred thymic cells (Figs. 1, 2). The optimal period for collecting the cells from the donor was determined by examining the antibody titres in the plasma of the recipients which received cells taken from immunized donors on days 3, 5, 7, 10, 14, and 21. Antibody productivity was most successfully conferred when cells were taken from immune donors 7 days after immunization (Fig. 1). In recipients, antibodies were detected within one day after transfer and the titre reached peaked levels on day 7 for mesonephric cells and day 14 for phronephric and splenic cells (Fig. 2). These results agree well with the kinetics of plaque-forming cells (PFCs = antibody-producing cells) and circulating antibodies described by Nakanishi (1987a). That is, PFCs were detected on day 3 and peak response was observed 5 to

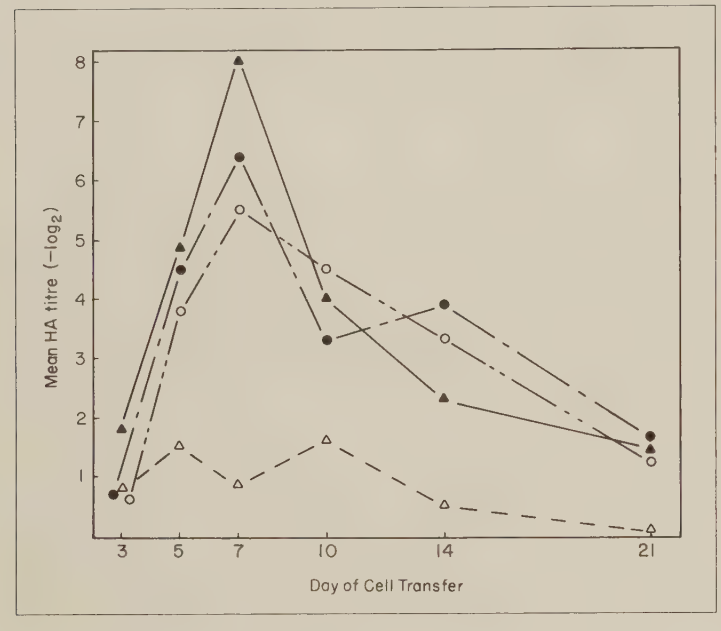

Figure 1
Kinetics of leukocyte transfer. Pronephros (●), mesonephros (○), spleen (▲) and thymus (△) cells taken from immunized donors on day 3, 5, 7, 10, 14 and 21 are injected into recipients. Each point represents mean hemagglutination titre in the plasma for 5 to 10 recipients.

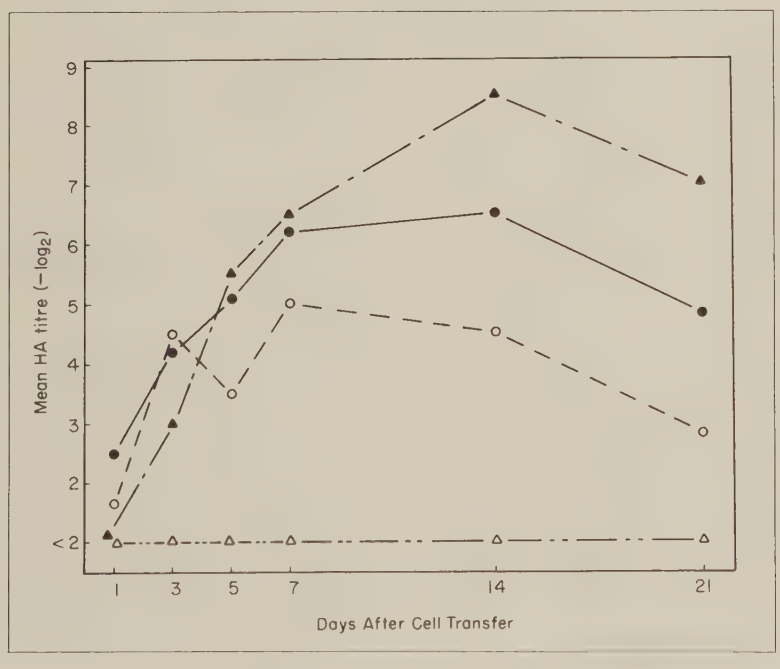

Figure 2
Antibody production kinetics. Recipients receiving 7-day postimmunized leukocytes from pronephros (●), mesonephros (○), spleen (▲), and thymus (△) are tested for antibody levels in the plasma. Each point represents mean hemagglutination titre in the plasma for 5 to 10 recipients.

7 days after the first injection. On the other hand, circulating antibodies were detected 5 days after the first injection and reached their peak on day 14. In the present study transfer of thymic cells was not successful, even though the number of PFCs of the thymus was similar to that of the pronephros and the spleen. This findin g leads to the idea either that thymic PFCs are not the cells involved in the transfer of immunity or that thymic cells need collaboration with other sensitized lymphoid cells to produce antibodies. In addition, no direct relationship between the PFC response and transferability for individual fish was observed in other lymphoid tissues (Nakanishi 1987a). This lack of correlation between the PFC response and transferability might be attributed to the existence of different developmental stages or of heterogeneous populations of antibody-producing cells and the necessity of cell collaboration. In any event this basic infomation obtained by using isogeneic animals can be useful for further investigations of cellular immune mechanisms on fish immune systems.

Transferability of Immune Pronephric Cells in Isogeneic, Allogeneic and Xenogeneic Transfer Systems

The major histocompatibility complex (MHC) of mammals and birds consists of numerous genes involved in acute allograft rejection, cell collaboration, and cytotoxic lymphocyte effective functions (Klein 1977). In lower vertebrates, a single genetic region homologous to the MHC has been described in the anuran amphibian (Du Pasquier et al. 1975; Kaufman et al. 1985; Nakamura et al. 1986). The teleost is the lowest vertebrate in which a MHC is suspected to exist, owing to their vigorous rejection of foreign tissue grafts, though knowledge of a MHC in fish is sparse. The present study was undertaken to examine the transferability of antibody reactivity by immune pronephric cells among isogeneic, and xenogeneic crucian carp in order to analyze the correlation between transplantation antigens and determinants involved in cell collaboration or cell-mediated lympholysis.

In these experiments we used two clones of ginbuna (B1, D3) from Okushiri Island in the vicinity of Hokkaido Island and one clone (K1) from Lake Kasumigaura, Ibaragi prefecture. In addition, siblings of bisexual diploid kinbuna, *C. carassius buergeri* (formerly *C. auratus susp.*), were also used in hopes that some of them might be genetically related. Fish source and histocompatibility relationship among clones have been previously described (Nakanishi 1987b, Nakanishi and Onozato 1988). Cell transfer was carried out according to the methods described above. Scale transplantation techniques followed Nakanishi (1987c).

Interclonal transfer between strong H gene-disparate clones was carried out to determine transferability of immune pronephric cells. Transfer of antibody reactivity was not successful when pronephric cells were transferred from B1 to K1 clones, which differed in strong H gene as evidenced by the acute rejection of allografts. Little or no antibody titres were detected in the recipients 7 days after transfer (Table 4). On the other hand, interclonal transfer between weak H gene-disparate clones was successful. Antibody titre of recipients 7 days after transfer was similar to that of intraclonal (isogeneic) transfer (Table 4), although recipients rejected allografted scales from donors 1–2 months after grafting. Transferability of immune pronephric cells in xenogeneic transfer systems was also examined. Pronephric cell transfer was carried out from immunized kinbuna to unimmunized ginbuna. No antibody was found and all xenografted scales from kinbuna were rejected within 5 days in ginbuna (Table 4). These results suggest that transferability of immune cells is strictly controlled by "MHC."

The most interesting results were obtained when allogeneic transfer using pronephric cells was perfomed among siblings of kinbuna that had rejected allografts of each other in an acute manner. Two experiments were performed, each with different times of cell collection from the immunized donors: 7 days (Fig. 3A) and 14 days (Fig. 3B) after immunization. In each study, one of five recipients showed elevated antibody titre which peaked 2 and 3 weeks after transfer from the 7-day (Fig. 3A) and 14-day (Fig. 3B) postimmunized donors, respectively. Furthermore, one of the recipients with cells transferred from the 7 days postimmunization donor showed a fairly high level of antibody titre 5 weeks after transfer, while no antibody was detected one week and three weeks after transfer. These results suggest that the locus of "class II" antigens involved in cell collaboration or cytotoxic lymphocyte effecter function is limited in polymorphism in comparison to the transplantation antigens (class I antigens), because antibody reactivity was successfully conferred in some donor-host combina-

Table 4
Transferability of immune pronephric cells in isogeneic and xenogeneic crucian carp.

	Donor-host relationship	Weeks after transfer	HA[a] titre of recipients ($-\log_2$)					
Isogeneic transfer	B1 - B1	1	7.5	6	8	7	5	5
		2	6	6	8	9	6	—
Xenogeneic transfer	kinbuna - B1	1	0	0	0	0	1	0
		2	0	0	0	0	0	2
Inter-clonal transfer I[b]	B1 - K1	1	0	1	0	2	1	0
		2	0	0	0	0	0	0
Inter-clonal transfer II[c]	B1 - D3	1	9	6	7.5	4	4	4
		2	8.5	5.5	7.5	4	3	4

[a] Hemagglutination.
[b] Cells were transferred from B1 clone to a K1 clone that differs in strong H gene.
[c] Cells were transferred from B1 clone to a D3 clone that differs in weak H gene.

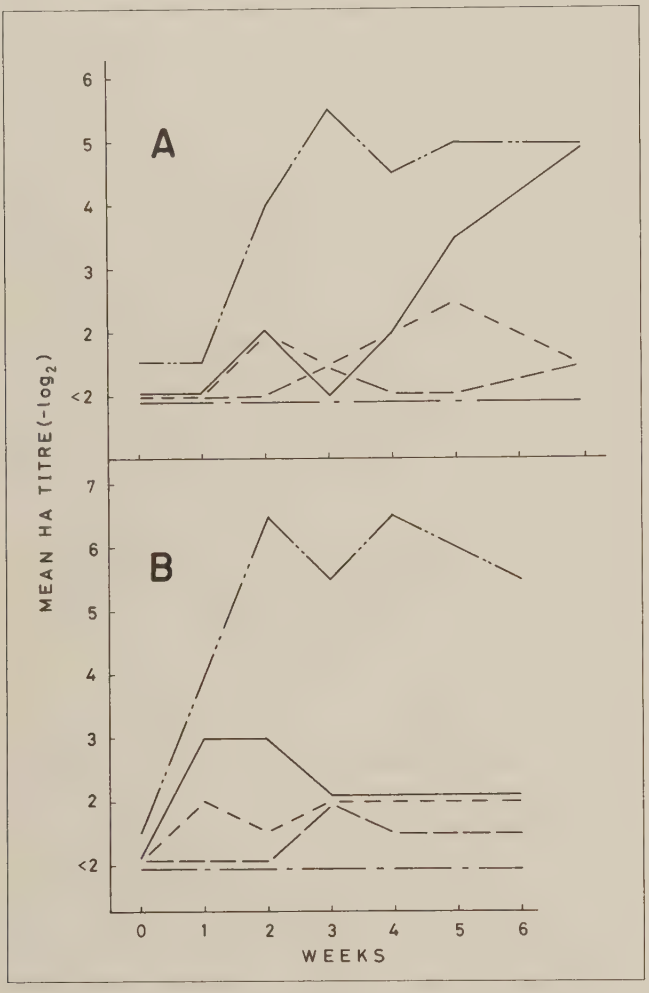

Figure 3

(**A**) Transferability of immune pronephric cells from donors 7-days postimmunization in allogeneic kinbuna crucian carp. Cells (5×10^6) per fish were intravascularly transferred and then antibody titres in the plasma of recipients were determined every week after transfer. Each line represents the antibody titre of individual crucian carp. (**B**) Transferability of immune pronephric cells from donors 14 days post-immunization in allogeneic kinbuna crucian carp.

tions even when the recipients rejected an allografted donor's scales in an acute manner.

Summarizing Comment

Many developments have contributed to the understanding of the immune system in fish over the last decade. However, more progress cannot be expected without using isogeneic or genetically defined models. Genetically identical animals offer many additional opportunities for analyzing the immune system of fish, as demonstrated with mice and rats. Indeed, clonal ginbuna are a useful tool from many aspects of fish immunology as shown in this review. There are of course some limitations for using ginbuna; one is the difficulty of crossing because of their gynogenetic reproduction and the other is their heterozygous state. Even so, ginbuna will continue to be one of the best models for the study of fish immunology until the establishment of inbred or clonal fish by means of chromosomal manipulation technique.

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Aquaculture of Striped Bass, *Morone saxatilis*, and Its Hybrids in North America[1,2]

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ABSTRACT

Increased interest has focused on the culture of striped bass, *Morone saxatilis*, and its hybrids (especially *M. chrysops*) as food fish with the recent decline in fishery landings of striped bass. Hatchery and pond culture techniques are sufficiently developed to allow initiation of farming operations throughout many areas of the southeastern U.S. Although aquaculture of these fish appears economically attractive, there are certain impediments to large-scale culture. Such impediments include issues of seed stock availability and cost, and restrictive laws and regulations. Such issues are being addressed and progress has been achieved in alleviating some of the impacts of these factors. It is expected that within the near future a sizable industry will be developed and that cultured striped bass and its hybrids will become readily available in many seafood markets and restaurants.

Introduction

Native stocks of striped bass, *Morone saxatilis*, have supported major fisheries in the United States along the Atlantic coast. However, since 1973 landings have declined dramatically (Fig. 1) and in an effort to protect the remaining stocks, commercial fishing bans have been implemented in many states. The striped bass is also an important recreational species and has been stocked in lakes, reservoirs, and rivers throughout the United States to support sport fisheries (Stevens 1984).

The striped bass is well known in the marketplace where it commands a high price (Sport Fishing Institute 1984; Swartz 1984). Because of the scarcity of this species and its high market value, interest has increased for culturing striped bass or a suitable market substitute. During recent years, research and development activities have included attempts to rear striped bass and its hybrids in ponds, netpens, raceways, and tanks (Powell 1973; Valenti et al. 1976; Wawronowicz and Lewis 1979; Williams et al. 1981; Kerby et al. 1983a, b; Woods et al. 1983, 1985; Collins et al. 1984; Kerby et al. 1987; Smith et al. 1987). Results have been highly encouraging and now there is commercial interest in many states to rear these fish for market (Smith and Jenkins 1985a).

This manuscript reviews and discusses the various considerations associated with aquaculture of striped bass and its hybrids in North America. In particular, information on broodstock acquisition and culture, spawning, hatching, larval rearing, and production data are presented. Also, comparative performance data on some of the various hybrid striped bass crosses are included.

Status of Aquaculture

Supply of "Seed Stock"

A basic impediment to the large-scale development of striped bass and hybrid bass farms has been the lack of a dependable supply of seed stock (Joint Subcommittee on Aquaculture 1983; Smith and Jenkins 1985b; Smith 1987). Presently, state and federal hatcheries rely on the capture of wild adults from spawning grounds to support hatchery operations (Harrell 1984). However, the private sector is typically prohibited from collecting broodstock using the same techniques (especially electrofishing) and from obtaining stock from areas used by these public hatcheries. Consequently, acquisition of ripe broodstock by the private sector is unpredictable, inefficient, and highly regulated. In recent years, there has also been an overall decrease in

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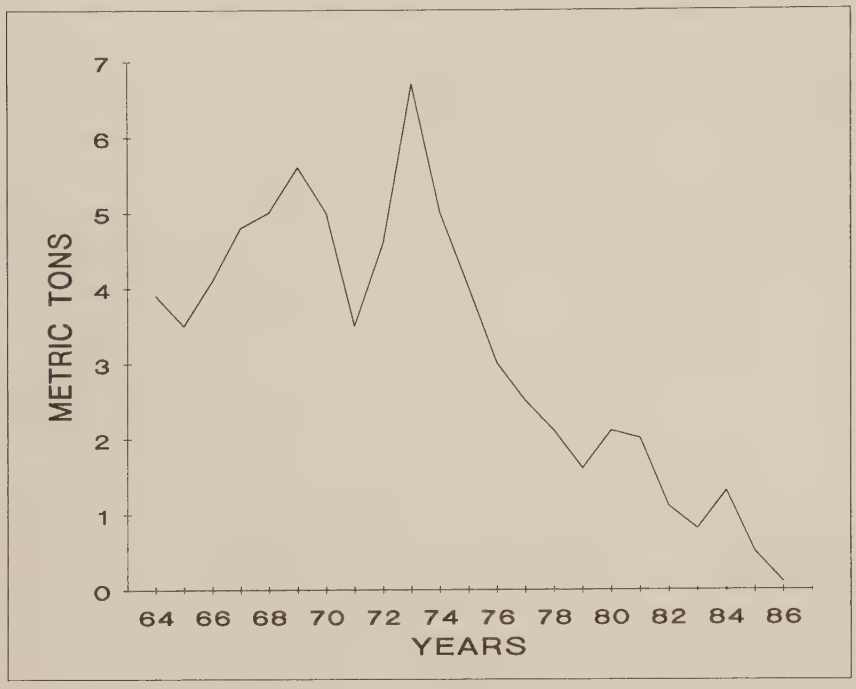

Figure 1
Commercial landings of striped bass on the Atlantic coast. (Fisheries of the United States, formerly Fisheries Statistics of the U.S.)

broodstock numbers such that even state and federal hatchery managers are currently experiencing difficulty in meeting their production goals.

Broodstock Development Research

In order to alleviate this "seed stock" problem, South Carolina researchers began in 1982 to examine the feasibility of developing domesticated broodstock to support hatchery operations (Smith and Jenkins 1984). Progeny of wild striped bass were reared in tanks and subjected to controlled temperature and photoperiod regimes (Smith and Jenkins 1986). During a five-year period the growth, maturity, and spawning success of these fish were monitored. At 33 months of age males had attained a size of 2.3 kg and essentially all were mature (Table 1). However, at 33 months of age only 25% of the females were mature and difficulty was experienced in spawning these fish (Smith and Jenkins 1986). During the following two years additional females matured while no additional mature males were observed (Table 1).

Spawning of wild striped bass broodstock is accomplished by injecting the newly captured fish with human chorionic gonadotropin (HCG) at a rate of 330 I.U./kg. Then, the fish are either "naturally" tank spawned by pairing up males and females in a tank (Bishop 1975), or the eggs are stripped from the female at time of ovulation and artificially fertilized with stripped milt (Bayless 1972; Bonn et al. 1976). In the first case, larger facilities are needed to house spawning tanks but effort is less intensive and damage to broodstock is reduced. In the latter method, the space required is reduced but effort is intensive and often broodstock die as a result of handling and stripping procedures. In South Carolina, both techniques have been used with 3- to 5-year old cultured broodstock. It is recommended that the "tank spawning" method be employed with striped bass in order to reduce fish injury. Although substantial progress has been achieved, the culture techniques developed for striped bass females need additional research to improve the predictability of spawning success. In contrast, development of domesticated male broodstock has been highly successful. Males can be produced in 2 to 3 years and used over several years (Smith and Jenkins 1986).

Table 1
Age, size, and percent maturity for cultured striped bass broodstock (updated from Smith and Jenkins 1986).[a]

	Males		Females	
Age (Months)	Mature[b] (%)	Weight (kg)	Mature[c] (%)	Weight (kg)
21	22	—	0	—
33	100	2.3	25	3.4
46	100	3.9	75	4.9
60	100	5.2	100	6.4

[a] Based on number of males and females at 60 months of age. 19% of the fish did not mature by age 60 months.
[b] Fish which expressed milt.
[c] Fish with eggs greater than 700 μ in diameter.

The techniques described above are used for the production of striped bass fry. However, the striped bass is not the preferred fish for an aquaculture operation because of its slower growth during the first two years and its reduced environmental tolerances. Concurrent research conducted with the striped bass and white bass (*M. chrysops*) crosses has indicated that these hybrids are the preferred fishes for aquaculture development (discussed later in text). In order to make "original cross" hybrids, female striped bass and male white bass must be stripped because they can not be induced to tank spawn together. Consequently, the problems of acquisition, predictability, and reuse of female striped bass occur. However, the "reciprocal cross" (female white bass × male striped bass) can also be performed which results in a hybrid which is quite satisfactory for aquaculture development (see later section). White bass are smaller than striped bass at maturity (minimum size for females ~300 g vs. ~3.4 kg for striped bass) and this species is abundant throughout many areas of the U.S. Although this species is of recreational importance, there is much less public sentiment associated with their removal for private hatchery use compared to the collection of striped bass (especially females).

Broodstock development activities with white bass have been based on the use of wild-caught fish, although cultured white bass may also prove to be suitable. Researchers in South Carolina have demonstrated that adult wild fish can be captured in the fall, matured in outdoor tank culture systems using ambient conditions, and spawned in the spring (Smith and Jenkins 1986). Unlike striped bass, adult white bass are either ripe males or ripe females in the spring with few or no fish of unknown sex. Work-to-date indicates that white bass females and striped bass males are easy to spawn and will naturally mature together in outdoor tanks (Smith and Jenkins 1985b, 1986, 1987). HCG is also used with white bass but it is administered at a higher level (750–1500 I.U./kg). Average production of reciprocal cross hybrid bass fry is in the range of 35000 to 90000 per female fish 460 to 640 g in weight (Smith and Jenkins 1986; Table 2). Recent demonstration of these spawning techniques using captive, wild broodstock should enhance the development of hybrid striped bass aquaculture farms (Smith and Jenkins 1987).

Table 2

Summary of reciprocal cross fry production using captive wild white bass females and cultured striped bass males (updated from Smith and Jenkins 1986).

Trial No.	Fish Weight (kg)	Larvae Hatched (no.)
1	0.63	33992
2	0.46	37247
3	0.64	84185
4	0.61	17870
5	0.64	87950
6	0.68	48100[a]
7	0.73	48100[a]
8	0.65	48100[a]
9	0.66	48100[a]

[a]All hatches were pooled, 192000 fry produced.

Incubation and Hatching

Eggs of striped bass and its hybrids are typically incubated in McDonald hatching jars (Bonn et al. 1976). Depending on egg type, approximately 100000–250000 eggs are placed in each jar. Water injected through a center tube causes continuous upwelling and oxygenation, and eggs hatch in about 48 hours at temperatures of 18° to 20°C, (Bayless 1972). At time of hatching, fry swim up with the outflowing water and are concentrated in outside aquaria. In situations where striped bass are tank-spawned, eggs can be left in the spawning tank to hatch or the eggs can be collected and placed in McDonald jars. Striped bass eggs are semibuoyant and are easily kept rolling in the jars. In contrast, white bass eggs are highly adhesive and will readily form large clumped masses of eggs. Such clumping is undesirable as dead eggs are not easily removed and fungal infections can readily occur. In order to eliminate the adhesiveness, the fertilized eggs are placed in the McDonald jars and aerated in a tannic acid solution (150 mg/L) for about 10 minutes before clean fresh water is flowed through the jars. This technique reduces clumping and results in a higher hatch rate of reciprocal cross hybrids (Charles C. Starling, Florida Game and Fresh Water Fish Commission, Webster, FL 33597, pers. commun., March 1986).

Fingerling Production

The fry are reared in either freshwater or brackish water nursery ponds (Phase I) to a small juvenile size over a 30–45 day period (Bonn et al. 1976; Geiger 1983a, b; Parker and Geiger 1984). During the last 2–3 weeks of nursery rearing, dry feed is added to the ponds to serve as a replacement for the diminishing supply of zooplankton of a suitable size. Typical stocking density for the Phase I nursery ponds is about 250000 fry/ha. Survival ranges from 0 to about 80%, but 25% is common (Parker and Geiger 1984). Temperature in the ponds ranges from about 18 to 25°C. During 1985 and 1986, nursery trials were conducted at the S.C. Wildlife and Marine Resources Department's Waddell Mariculture Center (WMC). Production from these trials conducted at dissimilar densities was 281000/ha for striped bass and 110300/ha on the average for hybrids (Table 3).

The small juveniles are harvested by draining the pond and collecting the fish in catch basins adjacent to the drain

Table 3
Results of Phase I nursery trails with striped bass and its hybrids in brackish water (salinity 4–8 ppt) ponds.

	Stocking data			Harvest data		
Type of fish	Density (no./ha)	Date	Duration (d)	Density (no./ha)	Survival (%)	Mean wt. (g)
Striped bass	600000	4/28/87	30	281630	46.9	0.36
F_1 original hybrid	128000	5/08/85	41	96000	75.0	0.45
F_1 reciprocal hybrid	300000	3/10/86	57	74500	24.8	2.20
F_1 reciprocal hybrid	300000	3/10/86	56	161030	53.7	0.67

structure. Fish produced by private hatcheries are then graded and restocked in Phase II rearing ponds at a lower density or sold to other farmers. Indoor intensive nursery techniques using tanks have also been developed for Phase I rearing of striped bass and its hybrids (Lewis and Heidinger 1981; Kerby et al. 1983a; Carlberg et al. 1984; Smith and Jenkins 1984). However, production of juveniles from such tank nursery systems is often highly variable and the pond-rearing method is currently the preferred technique for Phase I production.

Performance Characteristics of Striped Bass and Hybrids

Hybridization studies with striped bass were initiated in 1965 after it was demonstrated that HCG could be used to induce final maturation and ovulation of striped bass eggs (Stevens 1966, 1967). The focus of the hybridization work was to develop a fish that had the growth and feeding characteristics of the striped bass and the environmental adaptability and less stringent spawning requirements of white bass (Bonn et al. 1976). The first hybrid produced was the "original cross." Later other crosses were made using white perch (*M. americana*) and yellow bass (*M. mississippiensis*) males as well as reciprocal and backcrosses (Bayless 1972). Early evaluation of these various hybrids was somewhat opportunistic and not well controlled. Nevertheless, from these studies it was concluded that the original cross hybrid was a good fish and stocking programs were initiated (Bishop 1968; Logan 1968; Williams 1971; Ware 1975). Field observations indicated that the hybrids outgrew striped bass during the first two years and were both easier to produce and hardier than striped bass.

Table 4
Summary of indoor tank studies examining performance characteristics of striped bass and its hybrids (updated from Smith and Jenkins 1985b).

	Fish stocked			Harvest data			
Type	Size (g)	Density (no./m^3)	Duration (d)	Size (g)	Survival (%)	Biomass (kg/m^3)	Feed conversion
			Study number 1				
Striped bass	4.4	31.6	287	289	96	8.8	2.3
F_1 original hybrid	6.4	31.6	287	507	100	16.0	2.2
F_2 hybrid bass	12.8	31.6	287	347	84	9.2	2.7
			Study number 2				
F_1 original hybrid	23.0	31.6	140	263	99	8.3	1.9
F_1 white perch hybrid	21.0	31.6	140	176	99	5.5	2.5
			Study number 3[a]				
Striped bass	6.0	29.2	56	34	89	1.4	1.7
F_1 original hybrid	13.2	29.2	56	77	98	3.6	1.6
F_1 reciprocal hybrid	10.9	29.2	56	57	93	2.5	1.9
Backcross hybrid	21.1	29.2	56	71	92	3.1	2.4

[a]Study is currently in progress.

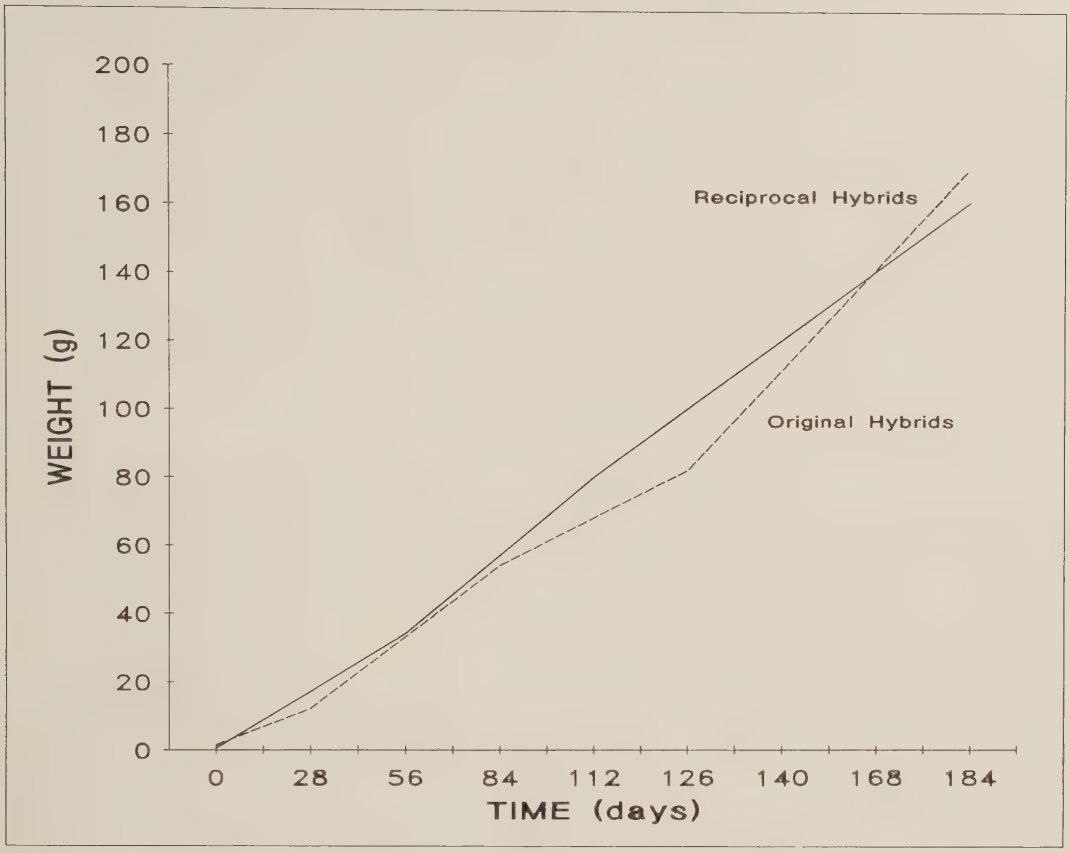

Figure 2
Comparison of F_1 original and reciprocal cross hybrids reared in ponds at a density of 10000 fish/ha.

With the increasing interest in aquaculture, more detailed performance information was desired for the various hybrids. Controlled studies were undertaken primarily in South Carolina and North Carolina. To date, not all striped bass hybrids have been tested but sufficient information is available to identify suitable candidates for aquaculture use.

A number of comparative studies have been undertaken by the Charleston Laboratory, S.C. Wildlife and Marine Resources Department, using indoor tanks which can recirculate either fresh or brackish water. In the first study striped bass were compared to reciprocal cross hybrids and to F_2 hybrids (F_1 original cross × F_1 original cross). Results of this 287-day study indicated that the reciprocal cross hybrid grew fastest and had a high survival rate (Table 4, Smith et al. 1985). In the second study the original cross hybrid was compared to a striped bass × white perch hybrid. Again, results indicated that the white bass hybrid grew rapidly and had a high survival rate (Table 4). In a current study, the original and reciprocal cross hybrids are outperforming the striped bass. However, a backcross hybrid (F_1 original × striped bass male) is also performing well at the present time and may be an additional aquaculture candidate (Table 4) (T. Smith, unpublished). Results of these replicated tank studies are also being corroborated in pond culture trials (Figs. 2, 3) (T. Smith, unpublished).

In summary, the hybrids of striped bass and white bass appear well suited for aquaculture. They can be raised in fresh or brackish water (Smith et al. 1986) and exhibit high growth and survival rates. Further, they can be reared in a broad range of water temperatures and are not killed by low (7°C) or high (33°C) water temperatures (Smith et al. 1987).

Grow-Out Production

Research data from production trials to produce market-size fish (≥568 g) have been limited although there is a commercial operation producing hybrids in tanks using geothermal water in California and another company is using net pens to produce striped bass in New York. Unfortunately, production data from these two operations are proprietary. In 1984, North Carolina researchers harvested grow-out trials in 0.1 ha earthen ponds using original cross hybrids. Survival averaged 83.9% and production ranged from 5247 to 5765 kg/ha (mean 5504 kg/ha) (Kerby et al. 1987). Recently, the S.C. Wildlife and Marine Resources completed its first pond grow-out trial using original cross hybrids. This study was conducted in a 0.5 ha pond at WMC. One-year-old juveniles (mean size 220 g) were stocked in March 1986 at a density of 12000/ha for final

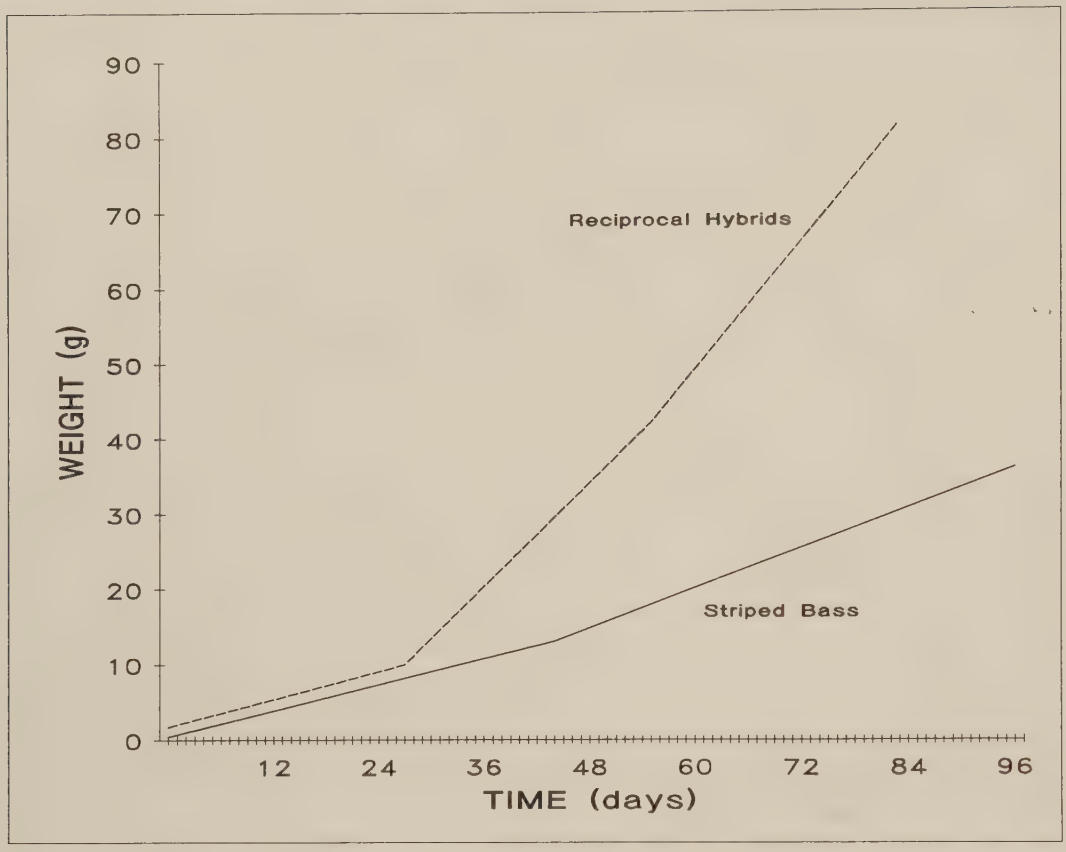

Figure 3
Comparison of striped bass and reciprocal cross hybrids reared in ponds at a density of 37000 fish/ha.

grow-out to market size. During the growing season (April–November), fish were fed a commercial trout pellet twice daily and satisfactory water quality conditions were maintained with paddlewheel aerators and water exchange. From December, 1986 to January, 1987 the fish were harvested and marketed. At harvest, mean fish size was 755 g and survival was 93% (Table 5). Total production was 8323 kg/ha with 93% of the fish ⩾568 g (1¼ lb). These results are highly encouraging and represent the highest pond production level yet achieved. Currently, a pond grow-out trial is underway with reciprocal cross hybrids and growth to the present appears similar. From an aquaculture perspective, routine production levels of 3900 to 7800 kg/ha should be attainable using techniques similar to those employed for producing channel catfish (*Ictalurus nebulosus*).

Market Testing

The opportunities and constraints associated with the marketing of hybrid striped bass were recently examined by Carlberg and Van Olst (1987). Prices received for the cultured hybrids have been in the range of $5.50–11.00/kg depending on product type (e.g., iced, gutted, live) and specific market (e.g., wholesaler, retailer, restaurant). Organoleptic testing of pond-reared cultured hybrids has been conducted by the Southeast Fisheries Center [National Marine Fisheries Service (NMFS)], Charleston, S.C. Results indicate that the hybrid striped bass is a mild flavored fish which should have good market appeal (Michael Jahncke, NMFS, Charleston, SC 29412, pers. commun., June 1987). Restaurant testing has also been conducted using our pond-reared hybrid bass. Again,

Table 5
Stocking and harvest data for pond grow-out trial using original cross hybrid striped bass.

Stocking data					Harvest data			
Density (no./ha)	Mean wt. (g)	Age of fish (d)	Pond size (ha)	Duration (d)	Mean wt. (g)	Survival (%)	Production (kg/ha)	Feed conversion
2000	220.2	327	0.6	240	755	93	8323	1.6

Table 6
Consumer evaluation (N = 216) of hybrid striped bass served in two Flordia restaurants (adapted from Liao et al. 1987). Rating scale: 1 = poor, 2 = fair, 3 = good, 4 = very good, 4 = excellent.

			Evaluation catergories					Consumer repurchase (%)		
Restaurant	Entree	Price ($)	Appearance	Texture	Smell	Taste	Price	No	Maybe	Yes
A	Broiled	10.95	4.7	4.6	4.8	4.7	4.4	2	4	94
	Fried	10.95	4.2	4.7	4.2	4.2	4.1	22	0	78
	Grilled	10.95	4.4	4.4	4.4	4.3	4.3	8	0	92
B	Broiled	4.95	4.3	4.2	4.4	4.5	4.2	2	5	93
	Fried	4.95	4.1	4.2	4.1	4.4	4.2	2	5	93

results were favorable indicating that these fish are highly acceptable to consumers (Table 6, Liao et al. 1987). Thus, cultured hybrid striped bass appear to be an excellent market substitute for wild striped bass.

Aquaculture Constraints

At the present time, there are two major constraints to the development of striped bass farms: 1) seed stock availability and cost, and 2) laws and regulations. Recent research in South Carolina on the domestication and culture of broodstock has demonstrated commercially practical techniques for controlled production of "seed stock." Thus, in the near future, private hatcheries should be able to develop their own captive broodstock (especially striped bass males and white bass females) and thereby increase the predictability and availability of fry and small juveniles. This use of captive broodstock may also result in lower production costs and lower seed stock costs.

The legal issues have been a serious constraint in many states where fishery bans were instituted to protect native fishery stocks including game species such as striped bass. In most cases, there was no exclusion for farm-reared fish. During the past year there has been substantial interest expressed by the agricultural community, businessmen, and landowners, to commercially farm striped bass and its hybrids. Consequently, pressure has been exerted on legislators to legalize the culture and sale of these fish with the result that many states are currently re-examining their laws and making provisions for bass aquaculture (e.g., Florida, Virginia, North Carolina, Georgia, Mississippi). In addition to the laws concerning possession and sale of fish, there are many additional regulations which affect the aquaculturist. These deal with site permits, discharge constraints, use of medications, and broodstock collection techniques, among others (Jenkins 1987). Some of these laws and regulations are also being re-examined because of their impact on aquaculture development.

Conclusions

Commercial farming of striped bass hybrids is an emerging industry in the United States which will need additional research and extension activities to reach its full potential. Pond culture technology is currently available and is now in the process of being transferred to the private sector. Indeed, during the past year cooperative demonstration projects have been initiated in several states including Maryland and North Carolina. Within the next 3–5 years, pond culture operations are expected to develop throughout the southeast and middle Atlantic states as well as in California.

Although the research data base appears adequate for development of the industry, additional study is needed in the areas of 1) broodstock development and genetics, 2) identification of other suitable hybrids, 3) nutrition, 4) market development, 5) disease treatment and prevention, and 6) laws and regulations. All these areas can substantially impact the economics of bass aquaculture. For example, profitability could be improved by development of hybrids exhibiting faster growth through genetic breeding or manipulation (induced polyploidy) and by the identification of more cost-effective rations.

The future appears highly promising for the development of hybrid striped bass farms throughout many areas of the United States. For the most part, these fish will be reared as a high quality seafood product. However, it is also expected that these fish will be used to support recreational fee-imposed fishing operations as well. Such operations will provide income to the operator while at the same time affording recreational fishing opportunity to the public sector. In summary, the striped bass × white bass hybrids are not only important recreational fish but they also appear to be excellent candidates for commercial development.

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Computerized Image Analysis for Selective Breeding of Shrimp: A Progress Report

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ABSTRACT

Genetic improvement of aquaculture organisms will be an important component of future progress by the aquaculture industry. The benefits of selective breeding could be obtained more rapidly if high selection intensity could be applied. This will be practical with an approach based on computerized image analysis which can provide an accurate estimate of organism size. Preliminary studies were conducted with a computer assisted morphometric analysis which obtains more information on size and shape from an image of a shrimp body than more conventional direct measurement. Using computer digitization, a truss network of dimensional variables was collected from photographs of three species of adult penaeid shrimp and used in canonical discriminant analysis.This approach was found to provide more information for discrimination than a typically labor intensive conventional morphometric method. The results demonstrate the usefulness of two-dimensional images for selection or classification. A truss network data set was also collected from full-sib families of juvenile shrimp using the new method. This study shows the application of the technique to images from living animals in a format resembling selection. Phenotypic variation in size is analyzed by analysis of variance, and heritabilities of size based on principal component scores and single variables are calculated. The multivariate estimator of size had more of the variance in size attributed to family differences than most single variables (h^2 = 0.60 for PC scores vs. a mean of 0.49 for single variables). Ways in which computerized image analysis might be employed in selective breeding of shrimp are discussed.

Introduction

There has been much discussion about the possible use of high selection intensities for aquaculture animals. High selection intensities are possible because the very high fecundities of aquacultured organisms (e.g., penaeid shrimp) permit a very small fraction of the individuals to be used for replacement of broodstock. This paper will support several premises related to the use of high selection intensities in a broodstock selection program. First, the implementation of such selection intensities will be possible and should not result in genetic drift. Second, the labor intensive characteristics of such a program will benefit greatly from the use of computer image analysis, a valuable alternative to human observers. Third, obtaining accuracy in the selection process will require the use of multivariate procedures in the assessment of organism size.

If we compare the fecundity of shrimp with even the most fertile domestic animal, they are different by orders of magnitude. In commercial shrimp culture facilities, female *Penaeus vannamei* have been observed to average 60000 nauplii per spawn and 8–10 spawns during their productive life in a maturation facility (B. Ribelin, Laguna Madre Shrimp Farm, P.O. Box 4043, Los Fresnos, TX 78566,

pers. commun., June 1986). Let us use the figure of 600000 larvae from an average female and follow the offspring through a production cycle, assuming 50% survival in each stage. This percentage is low, but not outside the range of observed values. The numbers would change as follows: 300000 postlarvae, 150000 juveniles, and 75000 harvested subadults. Assuming a survival of 50% from selection to reproduction, one would only need to select four individual subadults to replace the original two breeders.

Selection intensity (i) is related to the response of a population to selection (R) by the simple equation $R = i\ \sigma_P\ h^2$ in which h^2 is the heritability of the trait under selection and σ_P is the phenotypic variance of the trait. The relationship between i and the proportion of animals selected (p) for truncation selection of a normally distributed trait is $i = z/p$, where z is the height of the normal curve at the truncation point (Falconer 1981). Thus the maximum selection intensity for the above example (based on a proportion of 4 selected from 75000) would be approximately 4.3, if all the survivors could be measured.

Lasley (1978) gives the percentage of the progeny required for broodstock replacement in domestic animals as 4–5% of males and 40–50% of females in beef cattle; 1–2% of males and 10–15% of females in swine and chickens. Such broodstock selection programs for domestic animals obtain selection intensities of 2.0 to 2.7 for males and 0.6 to 0.8 for females (Falconer 1981). With the very small proportion of the population that is required for replacement broodstock in aquaculture species, it is possible to achieve selection intensities of 3.0–4.0. Current selection programs at commercial shrimp farms select the top 1/2 to 1/3 of the size distribution. If we assume that the trait under selection is heritable, the predicted response under high selection intensity is 3 to 4 times higher than under the current system. This could be accomplished using within-family selection to remove the danger of bottlenecks caused by unrecognized restriction of the gene pool and inbreeding. The limitations on selection intensity are primarily logistical (i.e., high selection intensities would require major investments of personnel and time).

In a previous publication (Lester 1983), it was recommended that a single abdominal measurement, sixth segment depth (SSD), be used as a criterion in selection for size of penaeid shrimp (Fig. 1). Several preliminary selections have been performed using this character. A single human observer is capable of applying this criterion to 300–500 shrimp per hour, but not for many hours continuously (pers. observ.). Nevertheless, at a selection intensity of 3.0, one would be required to measure approximately 300 shrimp, about 40–60 minutes of work, to select a single potential broodstock. In order to obtain a broodstock of 3000, which is not large by commercial standards, the observers would have to work for over 2000 hours. A microcomputer imaging system could perform the measurements and make a decision on whether to cull or select in several seconds. Computer analysis would be limited by how fast the shrimp could be moved through the imaging system. A single unit could measure continuously and accurately for any amount of time necessary.

No matter what criterion is employed for the selection process, there will be an associated measurement error. At a high selection intensity, the variance due to measurement error could exceed the true phenotypic variance of the potential broodstock in the distribution. This would represent a serious problem for the classification of animals into potential broodstock and culls. This factor in misclassification can be reduced by moving from univariate to multivariate classification. Thus we are recommending that high intensity selection not be based on a single measurement such as SSD, but rather on a set of measurements.

The characteristic of commercial importance, rate of gain in size, is a latent variable which can not be measured directly in these selection programs. It can be estimated by analysis of multiple variables which are correlated to it. (See Bookstein et al. 1985, for a thorough discussion of multivariate estimation of the latent variable *size*.) In

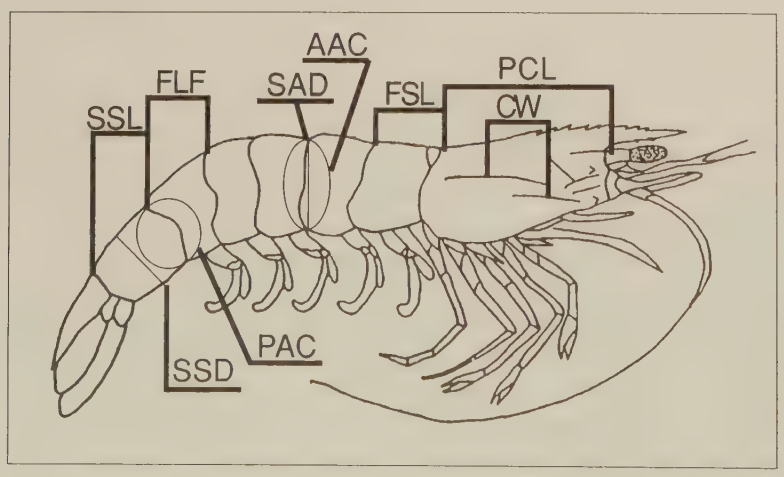

Figure 1

Diagram of shrimp with measurements used in the first study. Description of measurements: PCL = posterior margin of the orbit to posterior edge of carapace; CW = carapace width at the level of the last dorsal rostral tooth; FSL = first abdominal segment length; AAC = circumference at the intersection of the second and third abdominal segments; SAD = depth at the intersection of the second and third abdominal segments; FLF = fifth segment length with the abdomen maximally flexed; PAC = circumference at the intersection of the fifth and sixth segments; SSL = sixth segment length; SSD = depth at the midpoint of the sixth segment.

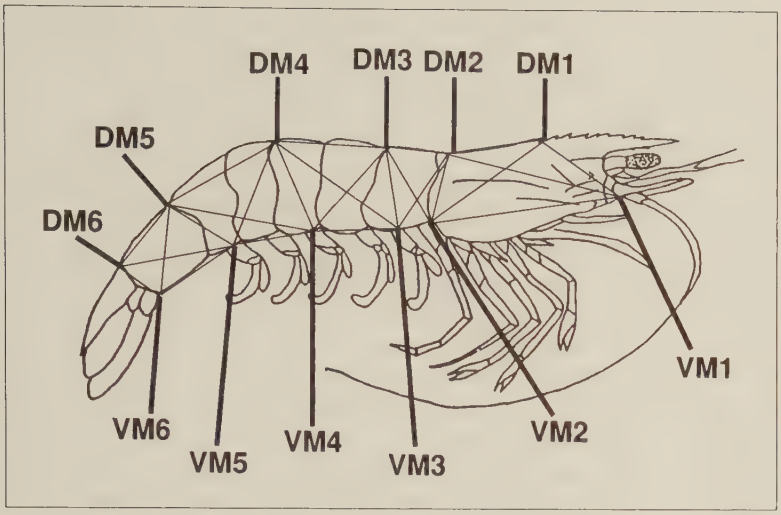

Figure 2
Diagram of shrimp with landmarks used in the adult truss network study. Descriptions of points: DM1 = Posterior rostral tooth; DM2 = dorsal, posterior point on carapace; DM3 = dorsal, posterior point on segment one; DM4 = dorsal, posterior point on segment three; DM5 = dorsal, anterior point on segment six; DM6 = dorsal, posterior point on segment six; VM1 = center of antennal basal segment at insertion; VM2 = center of basal segment of fifth periopod at insertion; VM3 = center of basal segment of first pleopod at insertion; VM4 = center of basal segment of third pleopod at insertion; VM5 = center of basal segment of fifth pleopod at insertion; VM6 = ventral, posterior point on segment six.

our approach to estimation of growth rate, the latent variable *size*, is represented by the first principal component (PC1) score obtained from a set of size-correlated dimensions taken from each shrimp. As variables are added, the variance-covariance matrix gets larger, but the variance of the multivariate mean (i.e., mean *size*) decreases according to the factor $1 - R^2$ (R^2 = the sum of the squared partial correlations between the variables). In other words, the multivariate data set becomes a better predictor of the underlying variable *size* when multiple variables correlated with *size* are used in the estimation process.

There is a school of morphometric studies centered around the work of F.L. Bookstein (Strauss and Bookstein 1982; Bookstein et al. 1985) which has developed an approach to the multivariate description of size and shape for fish. The approach is based on obtaining a set of variables that contains much information about specific regions of the body and is homologous across conspecifics of different sizes and across closely related species. This is accomplished by selecting landmark points on the morphology and arranging them into a set of boxes connected by all possible nearest neighbor connections into a truss network as shown in Figure 2 (Strauss and Bookstein 1982; Bookstein et al. 1985). The resultant network consists of a set of dimensional variables that covary with the latent variable *size* and contain information about shape-related deformational changes among a set of individuals. At the present time, we are interested in using this approach to precisely estimate genetic differences in *size*. Later we hope to apply it to the inheritance of shape.

One objective of the research program that we have started was the development of a system that would automate broodstock selection. This program would use a video camera to obtain an image that would be converted into digital format. This would be measured and analyzed by a microcomputer to obtain a summary of multiple variables which would permit the computer to decide whether the animal should be selected or culled. At the present time, we are still at an early stage in the development, using human observers to digitize still images.

This report deals with initial analyses of two data sets. These analyses are based on still images, not video, and only one uses living animals for the images. The first data set was obtained from adults carefully prepared for photographing to test the truss network approach on discrimination of *Penaeus* species. The second data set was collected from an experiment on growth differences among families of juvenile shrimp. It will be used to examine the way in which the partitioning of the variance in size is affected by changing from a single to a multivariate approach.

Materials and Methods

Testing the Truss Network Approach

Samples of adults were obtained from the following sources for both data sets: *Penaeus setiferus* from the wild near Galveston, TX; *Penaeus vannamei* collected from harvests of ponds at the King Ranch near Corpus Christi, TX, at the Laguna Madre Shrimp Farm near Harlingen, TX, and at Agromarina de Panama (only in the truss network data set) near Aguadulce, Panama; *Penaeus stylirostris* collected from harvests of ponds at the King Ranch near Corpus Christi, TX, and tanks at Marine Culture Enterprises near Laie, HI. The standard morphometric study used 104 *P. setiferus*, 92 *P. stylirostris*, and 134 *P. vannamei*. The truss network study used 49 *P. setiferus*, 42 *P. stylirostris* and 83 *P. vannamei*. All specimens were preserved by freezing and thawed for photography.

The standard morphometric data set contained nine dimensional variables and two weights, head weight and tail weight. Dimensional values were obtained with dial calipers except in the case of circumferences which were

obtained by passing a string around the shrimp and measuring the length of string with a ruler. The dimensional variables are shown in Figure 1. Data sets of this type have been obtained from six species of penaeid shrimp. Only the species *P. setiferus*, *P. stylirostris*, and *P. vannamei* will be used here for purposes of comparison to truss network data sets.

The truss network variables are shown in Figure 2. This data set contains 26 dimensional variables, no weights, and was obtained from pictures of the adult shrimp. The adults were positioned on a light table in the laboratory or on light plastic in the field for pictures taken at Agromarina de Panama. They were photographed with a scale in the frame using Kodak Tri-X black and white film. The negatives were mounted as slides and projected onto a digitizing tablet attached to a Tektronix graphics terminal. The operator used the cross-hairs of a mouse to locate 12 landmark points shown in Figure 2. Points were digitized in order from DM6 to DM1 to VM1 to VM6. The coordinates were stored in a data file, then converted to measurements by a Pascal program written by one of the authors (Wong, T.-C.).

All measurements were log transformed before use to remove the exponential effects of growth. Both the standard and the truss network data files were analyzed by multivariate statistical programs, PRINCOMP for principal component analysis and CANDISC for canonical discriminant function analysis (SAS Institute 1985). The principal component analysis was performed to obtain a summary estimate of the variation in the latent variable *size*. Discriminant function analysis was performed with the two data sets to illustrate the increase in information relevant to species differences contained in the truss network data set.

Multivariate Estimation of Heritability

Nauplii from ten separate spawns were shipped to the University of Houston - Clear Lake Aquaculture Genetics Laboratory from the Laguna Madre Shrimp Farm. Larvae were acclimated to Marine Mix sea water at laboratory temperature and stocked at 100 larvae/cone in three one-liter Imhoff cones per family. They were treated under standardized conditions: 28°C, 30 ppt, constant light, daily changes of water containing *Chaetoceros gracilis* at 100000 cells/mL and *Tetraselmis chuii* at 30000 cells/mL. After the protozoea-3 stage, *Artemia salina* nauplii were fed at 3/mL (Lester 1988). At the postlarva-2 stage, populations from those families with more than 120 survivors were transferred to 10 liter round cages. Three cages were stocked for each family with 40 postlarvae per cage. These 10 liter round cages had screened openings near their bottom and continuous water flow from the top. They were distributed among three large tanks (244 cm × 61 cm × 61 cm) which provided common water sources.

The postlarvae were weaned from *Artemia* to a prepared diet from the Texas A&M Shrimp Mariculture Project, and reared under the same temperature and salinity conditions for 40 days on a 14L:10D light cycle. The juveniles in each cage were fed once per day at ad libitum levels: 50 mg/cage in weeks one and two, 100 mg for five days, 200 mg for five days, 600 mg for five days, and, following sampling for survival and weight at 28 days, 400 mg to 8 cages with survival $<50\%$ and 600 mg to 14 cages with survival $>50\%$ for the remaining twelve days. Two cages had no survivors at 28 days.

After 40 days of growth, the shrimp were harvested and survival was determined. Six randomly sampled individuals from each cage were placed into a viewing cell filled with water and photographed using color slide film. Black and white film can be used and the negative mounted as a slide. Lighting is critical to the location of landmarks on small shrimp. Dual fiber optic illuminators positioned to minimize reflection from the exoskeleton were found to give better results than flash lighting. Slides were projected onto a digitizing tablet. The landmarks used were slightly different from those employed on the adults. The landmarks were changed from posterior rostral tooth (DM1 in Fig. 2) to ventral intersection of rostrum and carapace; from antennal insertion (VM1 in Fig. 2) to anterior ventral point on edge of carapace; pleopods marked at anterior edge rather than center point (points VM2 through VM5). The coordinates were converted to measurement variables by the same software and the log-transformed measurement variables were used in the SAS PRINCOMP program. Principal component scores were stored and ANOVA was performed using the SAS GLM program. Then the variance components were estimated with VARCOMP. Heritability was estimated from the variance components according to Becker (1984).

Results

Testing the Truss Network Approach

The correlation among the variables in the standard data set is high. Measurement PAC (Fig. 1) has the lowest average correlation of 0.68 and SSD the highest, 0.88. This indicates that these variables are related in their estimation of *size*. The first principal component (PC1) obtained for the standard data set represents the latent variable "*size*" as indicated by the equal loadings (0.28 to 0.35) of all variables (Bookstein et al. 1985). It explains 84% of the variance in the standard data set. The second principal component contains very little information, 0.05 of the variance which primarily represents the posterior abdominal circumference. The results from discriminant analysis can most easily be seen in the plots of canonical variate 1 (CAN1) against canonical variate 2 (CAN2). Figure 3 shows the plot resulting from analysis of the stan-

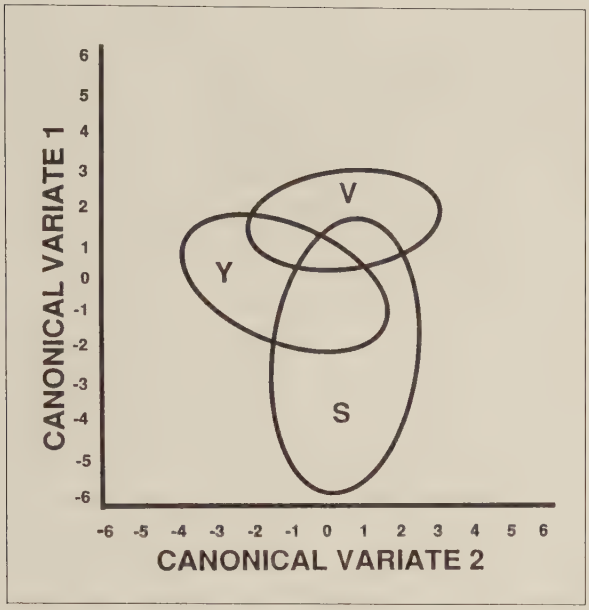

Figure 3

Plot of canonical variates 1 and 2 from discriminant analysis of the standard data set. The species are represented by ellipses based on the 95% confidence intervals of the species' centroids. The ellipse for *Penaeus setiferus* is labeled S, *Penaeus stylirostris* Y, and *Penaeus vannamei* V.

dard data set. The three species show some distinctness, but are not well separated. The Mahalanobis distances between the centroids of the three species are *P. setiferus* to *P. vannamei* = 3.69, *P. setiferus* to *P. stylirostris* = 2.51, and *P. vannamei* to *P. stylirostris* = 2.32. CAN1 in Figure 3 shows relatively high loadings of variables AAC (0.86), FLF (0.75), SSD (0.80), CW (0.73), and PAC (0.79) which could be interpreted as a body thickness variable. CAN2 shows high loadings of PCL (0.88), FSL (0.80), SSL (0.78), and SAD (0.85). It represents the variation in length of body segments among species. The goodness of fit of each discriminant variable to the classification variables (identifiers for species) can be evaluated by the canonical correlation which in the case of CAN1 is 0.84 and in the case of CAN2 = 0.56.

For the truss network data set, the correlation matrix indicated that the correlations were generally lower, and problems existed with one of the variables, VM2-VM3. This variable showed an average correlation with all other variables of 0.09. The remaining variables had average correlations ranging from 0.37 (DM4-DM5) to 0.69 (DM3-VM4). This data set gave results from principal component analysis that were different from the standard data set. PC1 explained 60% of the variation, PC2 explains 9%. All loadings on PC1 were between 0.15 and 0.23, except VM2-VM3 (0.02). Again this indicates that PC1 is a *size* component. The additional variables do not provide much allometric variation to be explained by PC2.

Table 1

Length of the sixth abdominal segment as an indicator of size differences among species and data sets.

	Data Set	
Species	Standard (cm)	Truss (cm)
P. setiferus	1.64	1.38
P. stylirostris	1.48	1.73
P. vannamei	1.87	1.56

At this point, there appears to be little on which to choose the truss network over the standard morphometric variables. However, results of discriminant analysis on the truss network data set are quite different as shown in Figure 4. This analysis results in larger Mahalanobis distances between the species' centroids, *P. setiferus* to *P. vannamei* = 5.94, *P. setiferus* to *P. stylirostris* = 6.35 and *P. vannamei* to *P. stylirostris* = 5.73. The discrimination of species along CAN1 is primarily due to values of the following variables (between group loadings given in parentheses): DM4-DM5 (0.84), DM4-VM5 (0.92), DM2-DM3 (0.95), DM5-VM5 (0.93), DM3-VM2 (0.97). There are 16 variables with between-group loadings above 0.75 on CAN2. Five are measurements from the carapace. Four are from the sixth abdominal segment; the remaining seven variables are measurements from the first, second, and third abdomenal segments. Thus CAN1 represents the first, fourth and fifth abdomenal segments and CAN2 the head and the second, third, and sixth abdomenal segments. The canonical correlations of these discriminant variables to the dummy classification variables (species identifiers) are higher than those obtained with the previous data set, for CAN1, 0.93 and for CAN2, 0.91. This indicates that the discriminant variables derived from the truss network approach are better for classifying individuals to species than those derived from the labor intensive approach.

It is unfortunate that these data sets are not the result of measurements on the same individuals. However, the sizes of animals in both data sets are comparable as indicated in Table 1 in which a variable contained in both data sets is compared (SSL in the standard data set is equal to DM5-DM6 in the truss network data set). There are also significant differences among the data sets. *P. setiferus* and *P. vannamei* samples are larger in the standard data set, but *P. stylirostris* are larger in the truss network data set. Size differences among the species could impact the discriminant analysis. In this case, the size differences among species are greater in the standard data set than in the truss network data set and should contribute to greater separation in the discriminant analysis of the standard data set. The discrimination is better however with the truss network data set, which only serves to reinforce

Table 2

Comparison of variance components and heritabilities obtained from nested ANOVA using simple variables and the first principal component scores. The eight simple variables were selected to represent lengths, depths, and diagonals, as well as the range of heritability estimates. Degrees of freedom in all cases: Family = 7, Cage = 16, and Error = 106.

Variable	Variance (family)	Variance (cage)	Variance (error)	Heritability
PC1	4.42×10^{-3}	0	1.04×10^{-1}	0.60
DM5-VM5	1.40×10^{-3}	0	4.44×10^{-3}	0.48
DM3-VM3	1.58×10^{-3}	0.05×10^{-3}	4.36×10^{-3}	0.53
DM2-VM2	1.92×10^{-3}	0.16×10^{-3}	4.66×10^{-3}	0.57
DM5-DM6	1.86×10^{-3}	0	4.85×10^{-3}	0.55
DM5-DM4	2.50×10^{-3}	0.36×10^{-3}	5.74×10^{-3}	0.58
DM3-DM2	1.72×10^{-3}	0.35×10^{-3}	5.56×10^{-3}	0.45
DM4-VM3	2.08×10^{-3}	0	4.56×10^{-3}	0.63
DM3-VM4	1.98×10^{-3}	0.15×10^{-3}	4.79×10^{-3}	0.57
Mean of 26 variables				0.49
SE				0.14

the conclusion that the truss network approach provides additional information about species differences.

Multivariate Estimation of Heritability

Examination of the correlation matrix output from the principal component analysis of the data from families grown under experimental conditions showed that two of the variables, VM2-VM3 and DM1-VM1 were weakly correlated with the remainder of the data set. The correlations of VM2-VM3 ranged from 0.25 (with DM1-VM1) to 0.66 (with DM2-VM2). For DM1-VM1, the correlations ranged from – 0.08 (with VM1-VM2) to 0.43 (with DM5-VM4). These ranges are distinctly lower than the other variables and are indicative of measurement problems. The juvenile shrimp correlations are higher than those in the truss network data set from the adults. PC1 explains 82% of the variance in the data set and has nearly equal loadings of all of the variables. The coefficients were between 0.16 and 0.23 which is indicative of their similarity as estimators of *size*.

Results from multivariate and univariate scoring were compared based on the heritabilities and variance components obtained from the same ANOVA design. Table 2 shows these results from comparison of eight univariate scores and the first principal component score. The results show that a univariate approach can yield inaccurate comparisons among genetic groups. Some variables have heritabilities that slightly exceed the estimation based on PC1. However, most variables yield lower estimates of the family component of the variance in *size*, some drastically lower. Using PC1 scores in the ANOVA gives an F value for the model of 4.74 and a heritability (h^2) of 0.60. The most extreme case is VM1-VM2 which has an h^2 estimate of 0. Clearly there are problems with this measurement. DM2-VM1 and VM2-VM3 have h^2 values of only 0.28. An average of all the h^2 estimates is somewhat lower than the one resulting from use of PC1 scores.

Discussion

Measurement problems are inherent in the development of a truss network design. In this study, they were reduced by making changes in the landmarks used and by improving photographic technique. Obtaining a simple set of landmarks that remain homologous across development from postlarva to adult proved more difficult than expected. One source of error in the adult data set was the choice of points on movable bodyparts, (e.g., appendage insertions on the adults). This problem was corrected by adopting new landmarks on the juveniles that represented stable intersections of body parts. In the case of the posterior rostral tooth, juvenile shrimp often expressed different degrees of rostral tooth development from conspecifics, both adults and other juveniles. Thus slightly different landmarks had to be used to collect dimensional data from juvenile shrimp of 0.2 to 1.0 gm than were used for the adults. When working with the juvenile shrimp, it was discovered that some points are more difficult to see on a live shrimp than on a dead one, (e.g., VM2). Better lighting and different photographic techniques were used to minimize this problem. The examination of different landmarks is continuing, especially for the juveniles.

Several variables used in the truss network analysis exhibited their unsuitability as *size* estimators by a reduced level of correlation with the other variables. The reason for low correlations from two of the variables, DM1-VM1

and VM2-VM3, was related to difficulty in precisely locating the landmark points, DM1, VM1, and VM2, on the pictures of juveniles. Some covariation is expected because all of the measurements are dimensions from the same image and all will increase with *size* of the shrimp. High correlation can be expected if all variables are reliable estimators of *size* and can be accurately measured. Variables in the truss network data sets obtained from juveniles were all reasonable estimators of *size* as indicated by the high correlations and equality of loadings on PC1.

The problems associated with developing a truss network approach are outweighed by the advantage obtained from it. In this study, the three species were used to represent any genetically distinct groups about which information is needed on distinguishing characteristics. It was shown that discrimination among *Penaeus setiferus*, *Penaeus vannamei*, and *Penaeus stylirostris* is possible using the standard data set as seen in Figure 3. However, discrimination among these groups improved with the truss approach, as one can see by comparing Figures 3 and 4. It appears that the truss network approach offers more information for the discrimination of these genetic groups and the classification of individuals than the collection of standard measurements. Comparison of a data set of nine variables and one of 26 variables for discriminant analysis is biased against the data set with fewer variables. Although Figure 4 shows that classification of individuals by species would be easier from a truss network approach, improved discrimination among species could perhaps have been accomplished by the addition of random variables to the standard data set. Thus these results should not be taken as sufficient justification for the truss network approach. A thorough justification can be found in Bookstein et al. (1985) and is based on information content and distribution within the data set.

The use of principal component scores derived from the truss network data set has associated advantages and disadvantages. The number of variables obtained is much larger than would normally be collected for a genetic analysis of size or any other characteristic. If these variables were to be measured by hand with calipers, the number of variables would prove prohibitive. The major advantage of using multivariate classification scores for the selection process will be realized in the improved accuracy of estimating *size* and reduced probability of misclassification. While this approach improves the analysis of size and shape of penaeid shrimp for experimental purposes, programming a computer to locate homologous landmark points with accuracy is a major challenge. Some other approach to image analysis may be better for commercial application in selective breeding.

There is considerable variation among the estimates of heritability obtained from univariate estimators of *size*. In some cases, it is not clear why more of the variance is partitioned into the family component, resulting in a higher heritability. It is clear that the range seen in Table 2 of

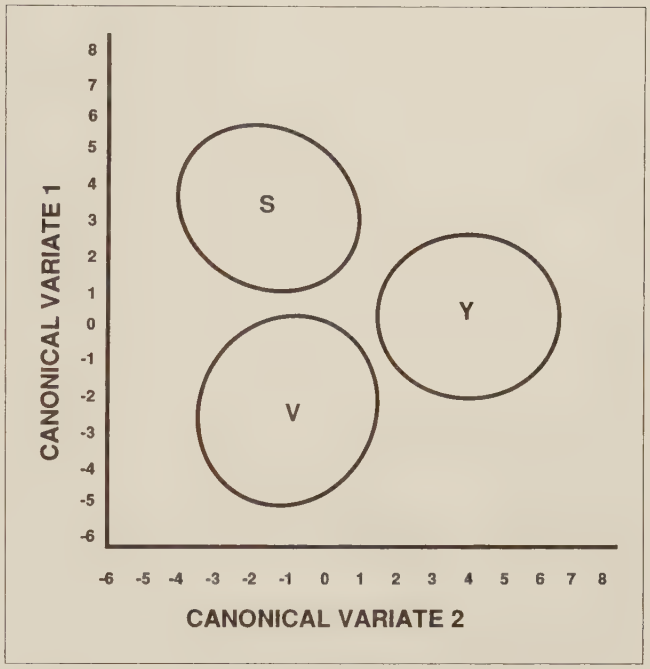

Figure 4

Plot of canonical variates 1 and 2 from discriminant analysis of the truss network data set. The species are represented by ellipses based on the 95% confidence intervals of the species' centroids. The ellipse for *Penaeus setiferus* is labeled S, *Penaeus stylirostris* Y, and *Penaeus vannamei* V.

0.45 (DM2-DM3) to 0.63 (DM4-VM3) is quite large for heritabilities of measurements taken from the same set of individuals from the same set of families by the same observers. This variation is indicative of differences in measurement error and information content among the variables in the truss network. Principal component analysis is one appropriate way to combine the information in many *size*-related variables and minimize the effect of measurement error. We believe that the use of multivariate classification analysis will provide a more reliable discrimination based on *size*. We will continue to pursue this approach in our experiments because it provides more accurate estimation of breeding value for *size*.

It is presently impossible to employ the image analysis approach for selection of untagged shrimp because the photography process and the computer analysis process are separated in time and space. Using our current digitizing process, it still takes the technician about two minutes to enter the landmark points from a single image. In addition, the time involved in taking good still photographs of living shrimp is considerable. These problems will be solved by the development of an integrated imaging and analysis system. The video image can be converted to a still image by a frame grabber which operates in 1/30th of a second. The ability to locate and record landmark points or to employ other image analysis techniques (e.g., definition

of the boundary of the shrimp and the area within that boundary) can be programmed into a fast microcomputer which could obtain the required measurements. The results of a preliminary classification analysis can be stored in the computer and used to calculate the selection index. With a sufficiently powerful computer, the calculation of the index and comparison to a truncation value would take a fraction of a second. Thus problems associated with the combination of many variables and too complicated an analysis could be solved by integration of fairly simple hardware and software.

Acknowledgments

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Breeding Test on Abalone

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Introduction

Although production of artificial seed for abalone farming in Japan has increased annually over the last 20 years, the total abalone harvest has continued to decline (Fig. 1) (Department of Statistics Information (DSI) 1967–86; Japan Sea-Farming Assoc. 1967–1985). In 1985 about 30 million seeds were produced. The proportion of recaptured artificial seedlings to the total abalones harvested in planting areas has usually been reported to be about 30–40%. However, some of these reports have shown values exceeding 90% (Table 1). In these cases it is important to study the genetics of the seed abalone used. Table 2 shows some examples of recapture ratios of abalone seedlings. Overall, the results suggest that seedling survival is usually low and a large percentage of the seed suffers mortality.

Another reason to study their genetics is the fact that differences in recapture ratios occur between species when they are planted on the same fishing ground (Inoue et al. 1985). Are there differences in the quality of the recaptured abalones compared to the seedlings which suffered mortality? In order to increase the recapture ratio, it is important to produce healthy seed abalone, improve seeding methods, and control the factors which affect survival on the planting ground.

Recapture Ratios and Seedling Quality

The survival rate within abalone species increases with seed size at the time of release. The extent of this increase, however, varies between species in relation to their size at maturity. *Haliotis gigantea*, for instance, is much larger than *H. discus* (Inoue et al. 1985) because its growth pattern (Takayama 1940) and feeding behavior is different (Momma 1980a). Thus, an increase in *H. gigantea* growth rate would need to be proportionally greater in order to have the same beneficial effect that a smaller increase would have on *H. discus*.

The faster growing seedlings of a *H. d. hannai* culture were recovered more frequently than the slow ones (Table 3). This higher rate of recovery illustrates that fast-growing seed have higher survival rates than their slower growing counterparts even though they were the same size at the time of release. It was previously reported by the author that juvenile abalone with initially rapid growth rates (until a shell length of 16–17 mm is reached) maintain that trait

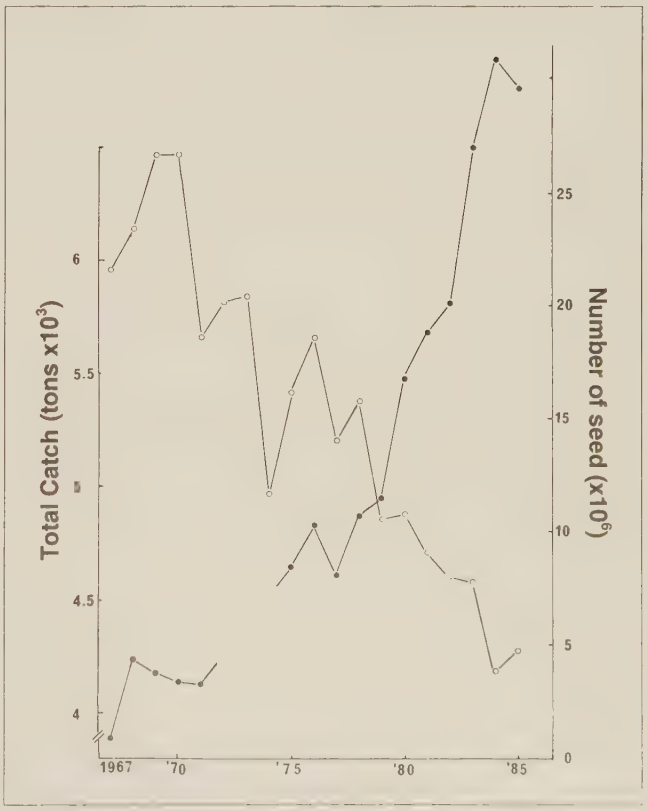

Figure 1
Total catch (○) and number of artificially produced abalone seedlings (●) in Japan. Total catch: after fisheries statistics of Japan 1967–86. Department of Statistics Information, Ministry of Agriculture, Forestry and Fisheries. Government of Japan. Number of seedlings: after Materials on production, supply and release of fingerlings for enhancement of fisheries resources in Japan, 1967–1985, Japan Sea-Farming Association.

Table 1
Proportion of artificial seed to abalone landed.

Prefecture	Location	Proportion of seedlings (%)	Species	References
Hokkaido	Toyohama	85	*H. d. hannai*	Momma (1986)
Iwate	Toni	39	*H. d. hannai*	Takeichi (1988)
Miyagi	Utatsu	83	*H. d. hannai*	Sasaki et al. (1987)
Fkushima	Nagasaki	59	*H. d. hannai*	Sato et al. (1984)
Kanagawa	Nagai	85	*H. discus*	Tauchi (1984)
Fukuoka	Oshima	54	*H. discus*	Futajima et al. (1985)

Table 2
Recapture rate of the planted seedlings.

Prefecture	Location	Recapture rate (%)	Species	References
Hokkaido	Shiribeshi	7	*H. d. hannai*	Miyamoto et al. (1982)
Iwate	Toni	18	*H. d. hannai*	Takeichi (1988)
Yamagata	Koiwagawa	26	*H. d. hannai*	Ioka (1983)
Ibaragi	Oarai	25	*H. discus*	Kodama (1985)
Kanagawa	Jogashima	26	*H. gigantea*	Inoue (1965)

Table 3
Recapture rate of juvenile *H. d. hannai* in its nursery ground (from Momma et al. 1980). Term of experiment was 116 days.

Group	Average growth ratio (μm/d)	Released size (mm)	Number released	Recovery	Recapture rate (%)
Fast	71	13.8 ± 1.3	964	170	17.6
Slow	28	13.9 ± 1.9	185	20	10.8

throughout their early development for a period of at least 116 days (Fig. 2) (Momma 1980b).

In this paper the effects of artificial selection, hybridization, and mutation on juvenile abalone growth are discussed. The isolated fast-growth qualities were examined through various tests conducted under standardized rearing conditions with the following results.

Growth Rates

The fastest growing individuals were selected from the offspring of one parent by mass selection when they were 150–200 days old: about 15 mm in shell length. After these superior individuals were reared about 1 more year, they were introduced for breeding and fertilized by the sib method. This method has been repeated several times from 1973 to 1989. The 6th generation of *H. discus hannai* produced by the sib method of inbreeding did not result in higher than normal mortality, or growth acceleration (Fig. 3) (Momma 1987).

H. discus discus and *H. discus hannai* were introduced for breeding by the usual method (Kikuchi and Uki 1974). These seedlings were reared under the same conditions. The growth and mortality for these species was compared. Similar shell-growth rates were observed for these two species, but mortality of the *H. d. discus* was higher than *H. d. hannai*; therefore, the latter produced a greater comparative biomass. (Fig. 4) (Momma 1987).

Hybridization studies were conducted on *H. d. discus*, *H. d. hannai*, and *H. kamtschatkana* using the usual method on the Ezo-abalone (*H. d. hannai*). In only one case did

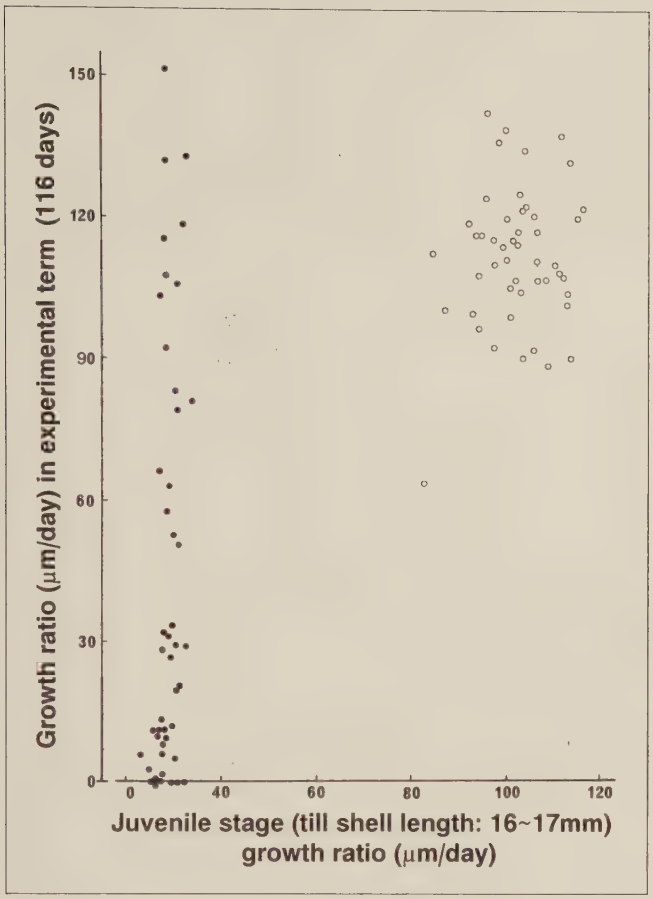

Figure 2

The relationship between the juvenile stage (shell length:16–17 mm), the growth ratio (μm/d), and the growth ratio during the experimental term (116 d). ○ = fast-growth group at 153–269 d old and ● = the slow-growth group at 570–686 d old (Momma 1980b).

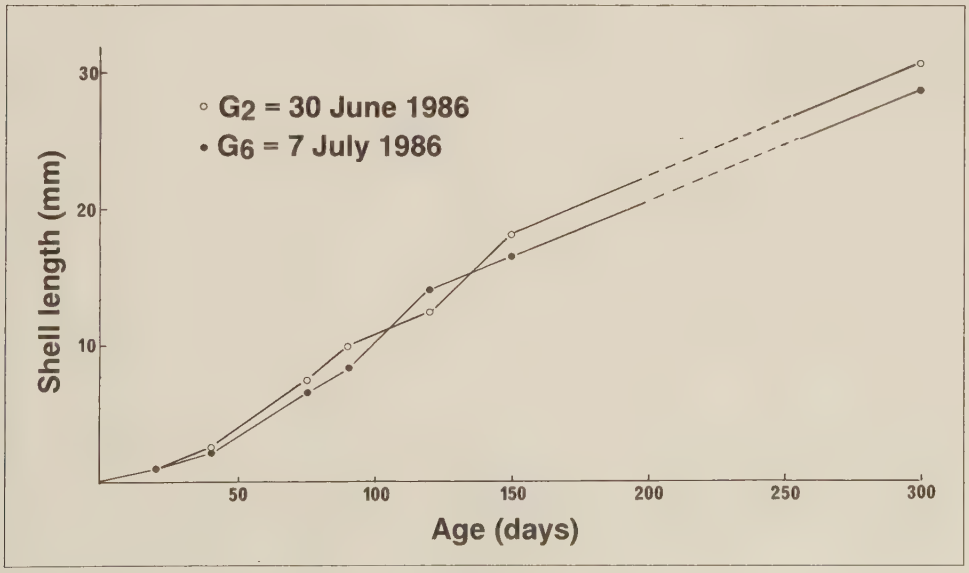

Figure 3

Growth lines of *H. discus hannai* seedlings; ○ and ● = the mean shell length of 2nd (G2) and 6th generation (G6), respectively. (G2 fertilized 30 June 1986; G6 fertilized 7 July 1986) (Momma 1987).

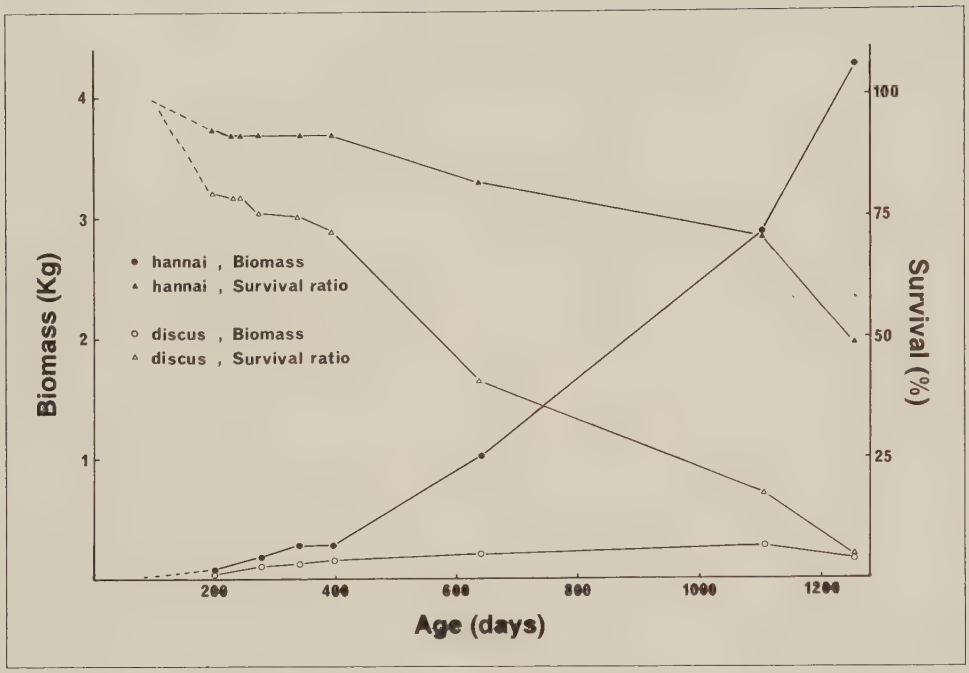

Figure 4
Biomass and survival rate of *H. d. hannai* and *H. d. discus*. (Momma 1987).

Table 4
List of the results from analyzed enzymes. (from Fujio 1984; Fujino 1979). D = digestive diverticula, M = shell-muscle, P = polymorphic, P* = polymorphic less than 5%, M = monomorphic.

Enzyme	Locus	Tissue	Polymorphism
Acid phosphatase	Acp	D	P*
Adenylate Kinase	Ak	M	M
Aspartrte aminotransferse	Aat	M	P
Esterase	Est-1	D	P
	-2	D	M
	-3	D	M
α-glycerophosphate Dehydrogenase	αGpd	M	M
Glucosephosphate Isomerase	Gpi	M	M
Isocitrate Dehydrogenase	Idh-1	M	M
Lactate Dehydrogenase	Ldh-1	M	P
	-2	M	M
Leucin Aminopeptidase	Lap-1	D	P
	-2	D	M
Malate Dehydrogenase	Mdh-1	M	M
	-2	M	P*
Malic Enzyme	Me	M	M
Mannosephosphate Isomerase	Mpi	M	P*
Octanol Dehydrogenase	Odh	D	P
6-phosphogluconate Dehydrogenase	6Pgd	M	P
PhosphoglucoseIsomerase	Pgi	D	P*
Phosphoglucomutase	m-1	M	P
	-2	M	P
Superoxide Dismutase	Sod	M	M
Tetrazolium Oxidase	To-2	D	P*
	-3	D	P*

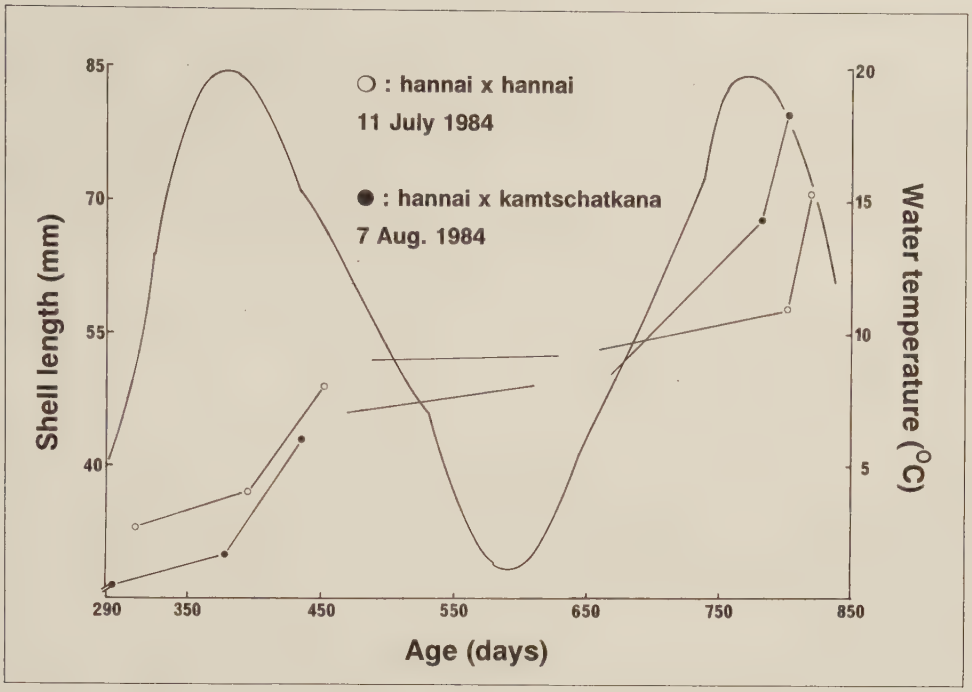

Figure 5
Growth rate in hybrid (●) and *H. d. hannai* (○) in the fishing ground. Curved line indicates the water temperature (Momma 1988).

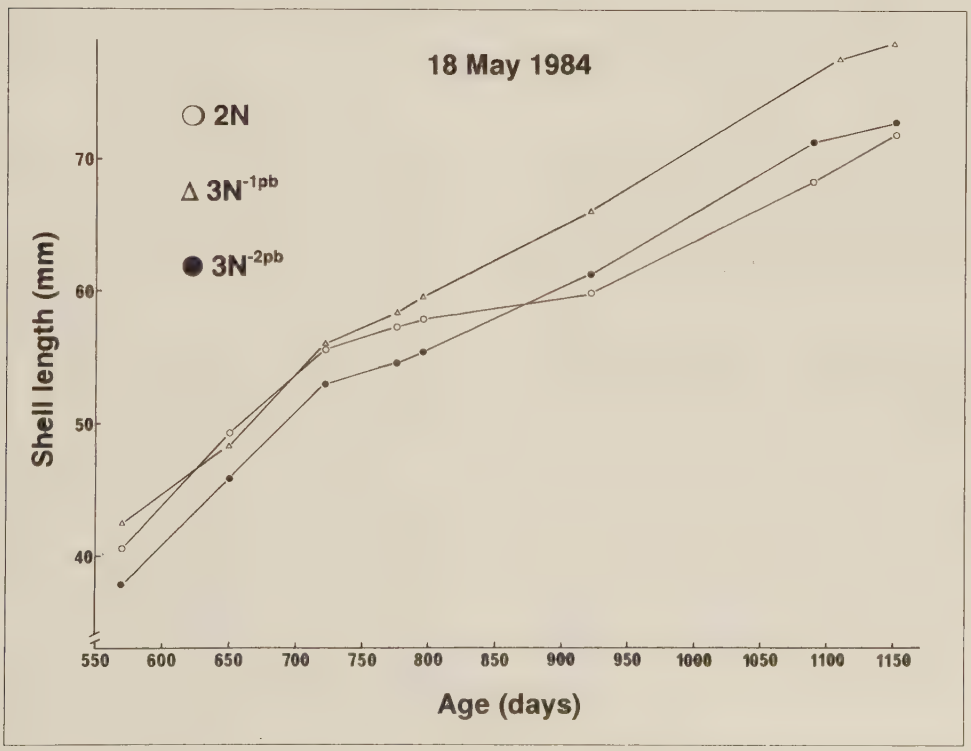

Figure 6
Growth rate in triploid and normal diploid seedlings; ○ = diploid, △ = triploid produced by interference of 1st polar body, ● = triploid produced by interference of 2nd polar body, fertilized 18 May 1984, respectively (Momma 1987).

the hybrid of *H. d. hannai* and *H. kamtschatkana* show improved growth rate in cold water. (Fig. 5) (Momma 1988).

Triploids produced by interference of polar body projection (both 1st and 2nd polar body extrusions) using the method of Arai et al. (1982) did not differ in growth from diploid seed (Fig. 6) (Momma 1988).

Abalone Breeding

Enzyme polymorphism of this species (Table 4) (Fujio 1984; Fujino 1979), and the chromosome handling techniques and growth characteristics of other species have been reported. Above all, based on the study of wild abalone populations, as well as the relationship of age to fitness measured at the esterase M locus, the deficient animals reveal more homozygosity (Fujino 1978). Population genetics and thremmatological studies may contribute considerably to the abalone industry.

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Two-Stage Hybridization and Introgression for Improving Production Traits of Red Tilapias

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ABSTRACT

Two-stage hybridization and introgression were evaluated as breeding plans to develop improved hybrid populations of red tilapia. Both plans were successful in overcoming interspecific breeding barriers, thus allowing development of hybrid populations (both red- and normal-colored phenotypes), with a mix of desirable production traits. The hybrids were evaluated in replicated polyculture experiments involving blue tilapia, *Tilapia aurea*, channel catfish, *Ictalurus punctatus*, freshwater prawn, *Macrobrachium rosenbergii*, and Asiatic carps. Yield trials were conducted in earthen ponds. Two-stage hybridization ([*T. aurea* × red tilapia] × [red tilapia × *T. nilotica*]) was used to produce red- and normal-colored hybrids that grew to average weights of 313 and 292 grams respectively in 133 days. Under similar conditions (communal stocking at equal density), *T. aurea* controls averaged 252 grams. Introgressive breeding techniques ([*T. aurea* × red tilapia] followed by two generations of backcrossing red males [F-1 and F-2 generations respectively], to female *T. aurea*) were used to develop a cold tolerant tilapia hybrid with both red- and normal-colored phenotypes. After 146 days of culture, both the red- and normal-colored hybrids averaged 296 grams, while *T. aurea* controls averaged 276 grams. Irrespective of breeding plan, mortality of the red phenotype was significantly greater than either of the normal-colored phenotypes.

Introduction

Individual (mass) selection for rapid growth in the maternal mouthbrooding genus *Tilapia* has had limited success, indicating that the fraction of additive genetic variation is small relative to total genetic variation (Chan May Tchien 1971; Tiechert-Coddington 1983; Hulata et al. 1986). In such instances, hybridization and introgression can be utilized to avail for nonadditive sources of genetic variation (Brody et al. 1980).

Within the interfertile genus *Tilapia*, there are species which exhibit fast growth (*T. aurea*; *T. nilotica*), high fecundity (*T. mossambica*), cold tolerance (*T. aurea*), and salinity tolerance (*T. mossambica*; *T. aurea*). Also, red-colored mutant strains of *T. mossambica* and *T. nilotica* have arisen spontaneously from normal-colored populations (Fitzgerald 1979; McAndrew et al. 1988). Thus, a diversity of genetic resources is available for developing hybrids or synthetic breeds in which favorable traits are combined.

Several hybrid strains of red tilapia have been commercially produced and widely disseminated throughout Southeast Asia and the Western Hemisphere during the past fifteen years. Breeding plans used in developing these stains have not been well documented (Sipe 1979; Fitzgerald 1979). However, morphometric and electrophoretic data indicate common ancestry with a red-mutant strain of *T. mossambica* (Galman and Avtalion 1983; Halstrom 1984). The widely distributed U.S. "Florida strain," derived from the cross female *T. hornorum* × male *T. mossambica* (red mutant), has production traits similar to its parental lines: early sexual maturation, slow growth, and lack of cold tolerance (Halstrom 1984).

In replicated yield trials, Florida strain young-of-the-year fingerlings grew only 50% as fast as blue tilapia, *T. aurea*, (Behrends et al. 1982). Thus, it was hypothesized that production traits could be improved by hybridizing the Florida strain with *T. aurea* or *T. nilotica*, two of the faster-growing and later-maturing species. Subsequently, Behrends and Smitherman (1984) reported that cold tolerance and the red color trait could be incorporated into a single population via hybridization (female *T. aurea* × male Florida strain), followed by recurrent backcrossing of red hybrid males to *T. aurea* females.

This paper will present results of studies designed to evaluate the use of two-stage hybridization and hybridization followed by repeated backcrossing (introgression)

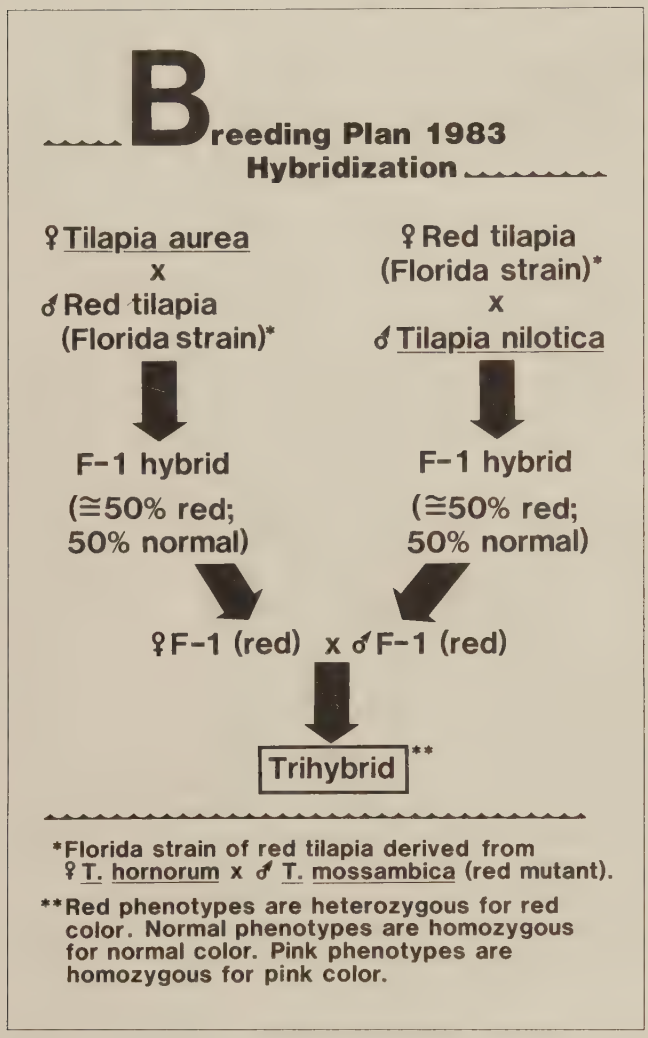

Figure 1
Breeding plan for developing cold-tolerant red tilapia hybrids.

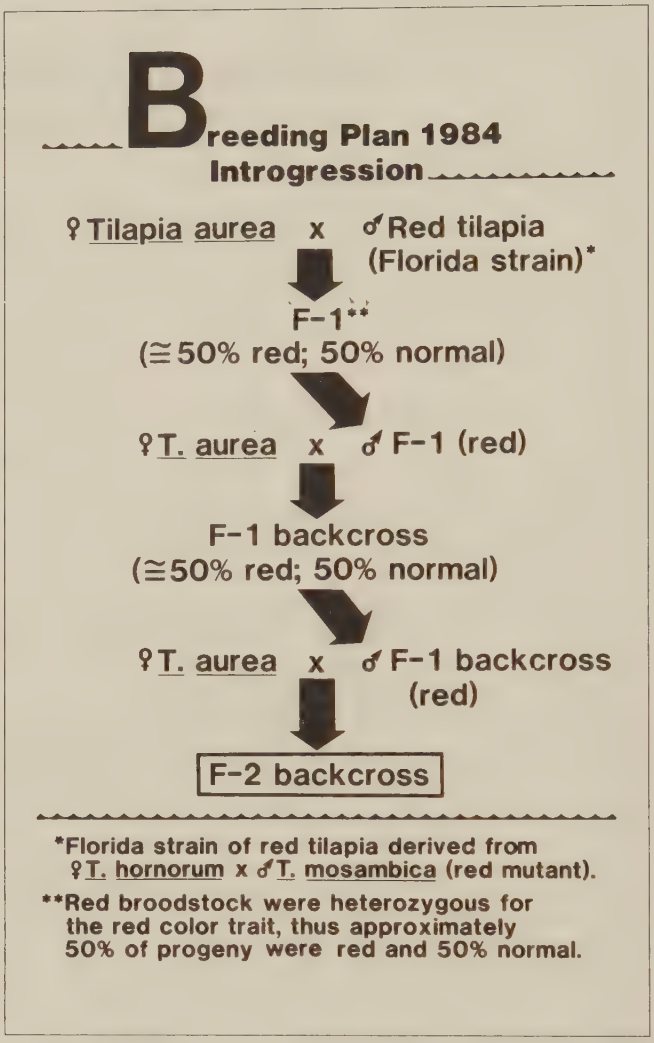
Breeding Plan 1984
Introgression
♀Tilapia aurea x ♂Red tilapia
(Florida strain)*
F-1**
(≅50% red; 50% normal)
♀T. aurea x ♂F-1 (red)
F-1 backcross
(≅50% red; 50% normal)
♀T. aurea x ♂F-1 backcross
(red)
F-2 backcross
*Florida strain of red tilapia derived from ♀T. hornorum x ♂T. mosambica (red mutant).
**Red broodstock were heterozygous for the red color trait, thus approximately 50% of progeny were red and 50% normal.

Figure 2
Breeding plan for developing fast growing red tilapia hybrids.

for developing red tilapia hybrids with improved culture performance.

Materials and Methods

Young-of-the-year red- and normal-colored (grey) tilapia fingerlings were produced at the Tennessee Valley Authority's Research Farm from 1981 to 1984 using breeding plans illustrated in Figures 1 and 2. In both trials, red broodstock heterozygous for the red color trait were used to ensure production of both red- and normal-colored phenotypes. By comparing the two hybrid phenotypes, it was possible to assess the effect of the red gene complex on growth, yield, and mortality.

In each of two years (1983 and 1984), three genetically and phenotypically distinct tilapia populations (Tables 1 and 2), were costocked into four 0.05-ha ponds along with channel catfish (*Ictalurus punctatus*, 10000/ha), freshwater prawn (*Macrobrachium rosenbergii*, 20000/ha), grass carp (*Ctenopharyngodon idella*, 120/ha) and F-1 hybrid Asian carp (*Aristichthys nobilis* × *Hypopthalmichthys molitrix*, 160–500/ha).

Fingerlings developed via two-stage hybridization were stocked in 1983, while introgressed hybrids were stocked in 1984. Red- and normal-colored hybrids and blue tilapia were costocked into each pond at equal densities (800/ha) for a total density of 2400/ha (Tables 1 and 2). Blue tilapia served as within- and between-years controls. Costocking of different genetic groups, also referred to as communal testing, has been shown to be an efficient method for evaluating various tilapia strains and hybrids under conditions of common environment (Wohlfarth et al. 1983).

Throughout each study, a 32% crude-protein floating catfish ration was broadcast onto the pond surfaces daily. Feeding rates were adjusted daily based on projected catfish biomass (Behrends et al. 1985). After 130 to 140 days

Table 1

Stocking and harvest data for three tilapia populations costocked into 0.05-ha earthen ponds. Polyculture yield trial 1983, Tennessee Valley Authority, Muscle Shoals, Alabama. Each mean represents the average of four ponds.[a]

	Two-stage hybridization			
	Red hybrid	Normal hybrid	*Tilapia aurea*	
Stocking density (#/ha)	800	800	800	
Culture duration (days)	133	133	133	
Initial weight (g/fish)	2.0	2.1	2.3	
Final weight (g/fish)	313 a[b]	292 b	252 c	
Males (mean ± SD)	330 ± 63.0	318 ± 59.0	283 ± 38.0	
Females (mean ± SD)	256 ± 58.4	254 ± 57.0	225 ± 35.3	
Sex ratio (% males)	77	59	48	
Daily gain (g/fish/d)				
Males (mean)	2.5	2.4	2.1	
Females (mean)	1.9	1.9	1.7	
Survival (%)	88	93	100	
Yield (kg/ha)	219 a	216 a	200 b	Total 635 kg/ha

[a] Tilapias were stocked into polyculture ponds containing channel catfish (10000/ha) freshwater prawn (20000/ha) and Asiatic carps (300–600/ha) (Behrends et al. 1985).
[b] Means followed by different letters are significantly different ($P<0.05$, Student-Newman-Kuels test). Horizontal comparisons only.

Table 2

Stocking and harvest data for three tilapia populations costocked into 0.05-ha earthen ponds. Polyculture yield trial 1984, Tennessee Valley Authority, Muscle Shoals, Alabama. Each mean represents the average of four ponds.[a]

	Introgressed hybrids			
	Red hybrid	Normal hybrid	*Tilapia aurea*	
Stocking density (#/ha)	800	800	800	
Culture duration (days)	146	146	146	
Initial weight (g/fish)	0.6	0.9	0.9	
Final weight (g/fish)	296 a[b]	296 a	279 b	
Males (mean ± SD)	299 ± 65.2	325 ± 65.4	298 ± 63.4	
Females (mean ± SD)	268 ± 44.9	260 ± 62.0	254 ± 46.1	
Sex ratio (% males)	90	56	49	
Daily gain (g/fish/day)				
Males (mean)	2.0	2.2	2.0	
Females (mean)	1.8	1.8	1.7	
Survival (%)	65	92	71	
Yield (kg/ha)	153 b	217 a	156 b	Total 526 kg/ha

[a] Tilapias were stocked into polyculture ponds containing channel catfish (10000/ha) freshwater prawn (20000/ha) and Asiatic carps (300–600/ha) (Behrends et al. 1985).
[b] Means followed by different letters are significantly different ($P<0.05$, Student-Newman-Kuels test). Horizontal comparisons only.

of culture (May–October), ponds were drained and harvested. Tilapias within ponds were sorted by phenotype and sex within phenotype. Individuals were then weighed to the nearest gram.

Statistical models used to evaluate growth and yield data during 1983 and 1984 included effects due to phenotype, pond nested within phenotype, sex, and all first-order interactions. Student-Newman-Kuel's Multiple Comparisons Test was used to separate means (within years), at a significance level of $P<0.05$ (Barr et al. 1979). Details con-

cerning culture and yields of catfish, prawns, and carps are presented in a companion paper (Behrends et al. 1985).

Results

Two-Stage Hybridiation

Two-stage hybridization (Fig. 1), was an effective breeding strategy for enhancing growth rate and yield of red- and normal-colored tilapia hybrids (Table 1). After 133 days of culture, mean harvest weights (sexes pooled) of red and normal phenotypes were 313 and 292 g respectively. Red hybrids were heavier at harvest ($P<0.05$) than their normal-colored counterparts, indicating a positive growth effect of the red gene complex. In comparison, *T. aurea* controls averaged 252 g, and weighed less ($P<0.05$) than either of the hybrids. Irrespective of phenotype, males were 20 to 30% heavier than females ($P<0.05$). Relative ranking of mean harvest weights by phenotype were the same in all ponds, indicating that pond (environment) × phenotype (genotype) interactions were not a significant source of variation.

Net production of the three phenotypes ranged from 200 to 219 kg/ha for a combined yield of 634 kg/ha (Table 1). Mortality of red hybrids was consistently greater than mortality of normal-colored hybrids and *T. aurea*. Sex ratios (male:female) of red and normal-colored hybrids averaged 77% and 59% males respectively, while the *T. aurea* population averaged 48% males: near the expected 1:1 sex ratio.

Despite their higher rates of mortality, hybrids yielded 7–10% greater than *T. aurea* ($P<0.05$). Improved yields were due to improved growth of both males and females and male-dominated sex ratios.

Introgression

Introgressive breeding (Fig. 2), similarly improved growth performance of red- and normal-colored hybrids (Table 2), although not to the extent of two-stage hybridization. After 146 days of culture, mean harvest weights of both red- and normal-colored phenotypes (sexes pooled) were 296 and 296 g respectively. This differs somewhat from results of the 1983 study, where the red hybrid grew faster than it's normal-colored counterpart. At harvest, *T. aurea* controls averaged 279 g and weighed less than either hybrid ($P<0.05$). Irrespective of phenotype, males were 15–20% heavier than females ($P<0.05$). As in the previous years study, relative rankings of mean harvest weights (by phenotype) were the same between the four replicate ponds.

Net production of the three phenotypes ranged from 153 to 217 kg/ha, with a combined yield of 526 kg/ha (Table 2). Mean yield of the normal-colored hybrid was greater ($P<0.05$) than yields of the red hybrid and *T. aurea* owing to faster growth and excellent survival. Mortality of normal-colored hybrids averaged only 8%, while mortality rates for red hybrids and *T. aurea* averaged 35 and 29% respectively (Table 2). Sex ratio of the red hybrid population was highly skewed towards males (90%), while the percentage of males in the normal-colored hybrid and *T. aurea* populations was 56 and 49% respectively.

Discussion

Both two-stage hybridization and introgression were effective breeding strategies for enhancing growth rates of red- and normal-colored hybrids. However, relative to *T. aurea* controls, two-stage hybridization improved growth rates and yields to a greater extent than did introgression. This is not surprising because introgression, as practiced, is analogous to inbreeding; heterozygous hybrid populations become progressively more homozygous with repeated generations of backcrossing to a small founder population. Introgression is recommended for incorporating a desirable dominant trait from one population into a second, more productive population (Kirpichnikov 1981).

During both years, mortality figures for red phenotypes were higher than rates for normal-colored phenotypes. Elevated levels of mortality have also been reported in other production studies (Behrends et al. 1982, 1988), and in hatchery research (El-Gamal et al. 1988). This indicates that the red gene complex, while enhancing growth, may have had a negative pleiotropic effect on viability. Color mutations in green sunfish, *Lepomis cyanellus*, and common carp, *Cyprinus carpio*, have resulted in reductions in viability, growth or both (Dunham and Childers 1980; Kirpichnikov 1981). Other red mutant strains of tilapia have been identified (McAndrew et al. 1988; Tave 1989), and should be evaluated with respect to growth rate, viability, and use in hybrid breeding programs.

Breeding plans in this study worked well for combining traits of closely related species. While many species of maternal mouthbrooding tilapia are interfertile, commercial production of most F-1 hybrids is difficult owing to species-specific differences in breeding behavior (Lee 1979; Hulata et al. 1985). In the present studies, problems related to breeding behavior were overcome by resorting to double hybridization or introgression. In both breeding plans, each of the respective parents (F-1 and subsequent backcross generations), had a complete or nearly complete set of chromosomes in common. This condition was apparently sufficient to overcome natural reproductive isolating mechanisms which can impede reproduction between closely-related species. Similar interspecific breeding plans may be practical for developing unique hybrids or synthetic strains for special environments, for instance, a fast growing, salt-tolerant red hybrid for net-pen culture in tropical estuaries.

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Define all abbreviations, acronyms, and symbols in text, equations, and formulae. Abbreviate units of measure only when used with numerals or in tables and figures to conserve space. The numeral one (1) should be typed as a one, not a lower-case el (l).

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